MOLECULAR BIOLOGY OF ATHEROSCLEROSIS

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Hopkins, Paul N. Molecular Biology of Atherosclerosis. Physiol Rev 93: 1317–1542, 2013; doi:10.1152/physrev.00004.2012.—At least 468 individual genes have been manipulated by molecular methods to study their effects on the initiation, promotion, and progression of atherosclerosis. Most clinicians and many investigators, even in related disciplines, find many of these genes and the related pathways entirely foreign. Medical schools generally do not attempt to incorporate the relevant molecular biology into their curriculum. A number of key signaling pathways are highly relevant to atherogenesis and are presented to provide a context for the gene manipulations summarized herein. The pathways include the following: the insulin receptor (and other receptor tyrosine kinases); Ras and MAPK activation; TNF-α and related family members leading to activation of NF-κB; effects of reactive oxygen species (ROS) on signaling; endothelial adaptations to flow including G protein-coupled receptor (GPCR) and integrin-related signaling; activation of endothelial and other cells by modified lipoproteins; purinergic signaling; control of leukocyte adhesion to endothelium, migration, and further activation; foam cell formation; and macrophage and vascular smooth muscle cell signaling related to proliferation, efferocytosis, and apoptosis. This review is intended primarily as an introduction to these key signaling pathways. They have become the focus of modern atherosclerosis research and will undoubtedly provide a rich resource for future innovation toward intervention and prevention of the number one cause of death in the modern world.

I. INTRODUCTION

During the past 10–15 years, understanding of the molecular biology of atherosclerosis and related signaling pathways has progressed so rapidly that the field has become essentially unrecognizable to most clinicians, many students of the life sciences, and even veteran investigators in related disciplines, a lamentable knowledge gap that undoubtedly hampers progress in the field. For those interested persons who find themselves in this situation, this review is intended to fill a portion of that gap, which may have formed from lack of formal training, inattention to the field, or merely the passage of time.

Much of the text may be considered as an introductory primer or overview for a number of the signaling pathways now central to investigations into the molecular biology of atherosclerosis. Because the field is now so extensive, an in-depth review is precluded. While the listing of atherosclerosis-related genes herein is relatively exhaustive, the discussion surrounding each one is, of necessity, limited and focused on pathophysiology. Indeed, the purpose of the effort to find as many manipulated genes as possible was to identify relevant pathways and general mechanisms potentially related to atherosclerosis rather than to attempt an exhaustive review of the methodologies, treatment implications, or controversies. Indeed, only passing attention is given to treatments, molecular biology of standard atherosclerosis risk factors, or to results from human genetic association studies.

Reluctance to delve into the pathways presented herein may arise from their sheer complexity, the number of components, or failure to recognize their relevance, but perhaps also from the impression that the numerous abbreviations and sometimes fanciful names given to signaling molecules implies some kind of inherent transience in the field. Some of the most imaginative names come from fly genetics (e.g., Sos, son of sevenless; Boss, bride of sevenless; MAD, mothers against decapentaplegic; Shh, sonic hedgehog; Dbs, Dbl’s older sister where Dbl stands for diffuse B-cell lymphoma; Mam, master mind; Mib1/2, mind bomb 1 and 2). Historically, many names were given before much was known about the protein’s function or were based on the appearance of fruit flies with a mutation in the gene (such as Groucho). Commonly, only the abbreviation is communicated since the full name is frequently cumbersome and
shedding light on function (e.g., Keap1 is Kelch-like,
erthyroid-derived, cap-n-collar-homology-associated pro-
tein 1). Yet most of the signaling pathways related to
atherogenesis and presented herein are canonical, along
with their frequently odd names and abbreviations. Where
there is an alternative abbreviation or name suggested by
the HGNCC (HUGO Gene Nomenclature Committee), the
most commonly encountered abbreviation is generally
used. Further details regarding individual signaling compo-
nents, protein structure, domain arrangements, and activa-
tion mechanisms are available in recent texts (634, 947,
1141) or a notable on-line, freely accessible textbook of cell
signaling (136) (http://www.biochemj.org/csb/).

While considering a number of these pathways, this re-
viewer was struck by one remarkable feature: redundancy.
In a redundant system, a duplicate or similar element may
take over the function of another element or provide a
somewhat different but overlapping utility. Often built into
cleverly designed mechanical or informatics systems, redun-
dancy can ensure the operation of critically important ac-
tivities even when one or more key components fail. In
living systems, the phenomenon of gene duplication, allow-
ing the original or duplicated gene to diverge somewhat or
greatly in function, provides a mechanism for redundancy
to arise naturally. Alternate transcriptional start sites and
particularly alternate splice sites are found in ~75% of
human genes and provide numerous isoforms with analog-
gous flexibility of expression (861). The multiplicity of cy-
tokines and multiple isoforms of various enzymes (such as
the thioredoxin and glutaredoxin systems) certainly illus-
rate redundancy, often showing overlapping if not entirely
duplicated functions. However, rather than simple linear
pathways with a few key back-up elements, the notion of
redundancy is taken to an entirely new level when consid-
ering the series of enormously interacting, branching net-
works that better describe living systems.

With the use of yeast as a eukaryotic test organism, a
remarkable 73% of 6,000 genes making up the yeast genome
were found unnecessary for survival (when tested one gene
at a time) and only 50% affected growth even in extreme
test conditions (613). When tested two genes at a time,
extensive interactive networks can be identified. One study
reported 117,000 significant interactions affecting yeast
growth out of 5.4 million potential interactions among the
1,700 genes tested. Informatics techniques allowed organiz-
ation of many of these apparent interactions into over-
lapping networks with known functional utilities (RNA pro-
cessing, mitochondrial activity, etc.) (352). In this study,
there were 39 interactions per gene on average and for only
10 genes were no interactions identified (C. L. Myers, per-
sonal communications).

Networks can act to buffer defects in single genes, distrib-
uting their effects to closely interacting network neighbors.

Networks may also broaden the functionality achievable by
any one protein, thereby providing substantial increases in
robustness and resiliency (429, 1107). Mathematically
defined network interactions may provide insights into unex-
pected relationships, such as the key role that HMOX1
(heme oxygenase 1, also abbreviated HO-1) was found to
play in the response of human endothelial cells to oxidized
phospholipid exposure (1501). The degree to which redun-
dancy or network behavior dilutes the effects of any one
gene on a complex trait like atherosclerosis is a particular
challenge to human genome-wide association studies (GWAS)
and related statistical methods of gene discovery.

Among the strongest effects on atherosclerosis in animal
studies was knockout (KO) of the gene CCR2 (chemokine,
CC motif, receptor 2) in the atherosclerosis-prone, ApoE-
deficient mouse (see FIGURE 1). CCR2 is the cognate recep-
tor for the ligand CCL2 (chemokine, CC motif, ligand 2),
also known as MCP-1 (monocyte chemoattractant protein
1). Other ligands for CCR2 (either in humans or mice)
include MCP-2 through 5, but these have been much less
studied in relation to atherosclerosis, although both MCP-1
and MCP-3 are required for monocyte mobilization from
narrow and homing to lesions. After just 5 wk of athero-
genic diet, there was a clear CCR2 gene-dose effect, as
shown in FIGURE 1. By 9 wk, however, any difference be-
 tween the heterozygous KO and wild type was gone. Fur-
thermore, there was only a 4-wk lag in the extent of athero-
sclerosis when comparing the strongly protected homozy-
gous CCR2 KO mouse to the wild-type mouse (compare
weeks 9 and 13, FIGURE 1) (179). Later studies tended to
show lesser percent differences, perhaps because of assess-
ments at later times or more aggressive atherogenic dietary
protocols.

Diminishing single gene effects with aging in studies such as
the above may be due to the multiple pathways and genes
that promote atherosclerosis in the face of severe hyperlip-
idemia (and probably other major risk factors for atherosclerosis). Surely, redundancy and compensation through networked interactions will greatly dilute measurable effects from any single gene, and such single gene effects will likely be less pronounced in the face of overwhelming risk factor exposure, such as severe genetic hyperlipidemia exacerbated by high-saturated fat, high-cholesterol diet.

Statistical modeling of gene-gene or gene-environment interactions may be more realistic but runs the risk of greatly reduced power and false positives due to the problem of multiple testing, even if interactions are restricted to known, physiologically defined networks. Undoubtedly, redundancy contributes greatly to the difficulty of discovering atherosclerosis-related genes by statistical methods in human populations.

While fascinating, genomic or proteomic approaches will not be emphasized in this review; rather, it will focus on individual genes and pathways. Included herein are all 166 unique genes listed in the Mouse Genome Informatics website listed under the phenotype “atherosclerotic lesions” (http://www.informatics.jax.org/, accessed March 6, 2013) as well as many more. Quantitative trait loci with no clearly identified causal gene are not included.

II. OVERVIEW OF ATEROGENESIS

Clinically manifest atherosclerosis may be viewed as the culmination of four major steps, a scheme adapted from the cancer literature and successfully used to classify risk factors for over 30 years (784). The steps include 1) initiation of endothelial activation and inflammation; 2) promotion of intimal lipoprotein deposition, retention, modification, and foam cell formation; 3) progression of complex plaques by plaque growth, enlargement of the necrotic core, fibrosis, thrombosis, and remodeling; and 4) precipitation of acute events. Acute clinical events, such as acute myocardial infarction (MI), unstable angina, ventricular fibrillation, or sudden coronary death are generally due to plaque destabilization and thrombosis but may also occur when acute ischemia is imposed on an electrically unstable myocardial substrate, precipitating ventricular fibrillation. As an illustration of precipitating risk factors, there is a clear increase in the risk of acute coronary syndromes when fighting fires (874), while shoveling snow after a snowstorm (843), or fighting fires (927), and as an example of the complexity of the atherogenic process, the risk of MI or CHD death was found to rise 21-fold during the 24 h following the loss of a spouse or other close loved one (1238) and 5.6-fold within the first week after the diagnosis of cancer (499). The associated risks for such events are clearly contingent on preexisting coronary artery disease (CAD).

In this scheme, major risk factors frequently act at more than one step of atherogenesis. For example, various forms of hyperlipidemia can contribute to endothelial activation (with or without oxidation of the lipoproteins) (1716), impair nitric oxide (NO) synthesis or availability (26), lead to foam cell formation (after a variety of possible modifications) (1732), increase platelet activation and thrombotic potential (e.g., oxidized phospholipids, found in excess in hyperlipidemic serum, interacting with platelet scavenger receptor CD36) (1426), and lead to reversible plaque destabilization (927, 1042). Obviously, factors that increase thrombotic potential may also help precipitate an acute event if they lead to an occluding thrombus. Factors that aggravate inflammation also associate with unstable plaque (1041).

Initiation occurs in predisposed areas where slow flow induces partial endothelial activation (especially when there is flow direction reversal, however subtle). Indeed, atherosclerosis essentially develops only in areas of slow or so-called “disturbed flow,” and mathematical models predict such areas precisely (940, 1343). In humans, vascular smooth muscle cells (VSMC) accumulate early at predisposed sites to cause intimal thickening prior to foam cell formation, possibly in response to platelet-derived growth factor (PDGF) production by activated endothelial cells (49). Indeed, the contribution of VSMC to multiple stages of atherogenesis may have been underappreciated in the past. Endothelial activation is further aggravated by other risk factors. Once endothelial cells are activated, initiation proceeds essentially as described for early stages of the innate immune response (1). Activated endothelial cells elaborate adhesion molecules such as E-selectin, P-selectin, and vascular cell adhesion molecule-1 (VCAM-1) and the chemokine MCP-1. Monocytes and probably neutrophils are among the early inflammatory cells to attach to the endothelium at first loosely then firmly and move into the subendothelial space. Monocytes transform into macrophages and are further activated by encounters with pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs), and various cytokines including tumor necrosis factor-α (TNF-α), interleukin (IL)-1, and IL-6. T-helper 1 (Th1) cells secrete interferon-γ (IFN-γ) and other cytokines to amplify the innate immune response. Concomitantly, PDGF (mostly dimers of A and B isoforms in humans) from adherent platelets and activated endothelial cells is released. Traditionally, PDGF was thought to act on differentiated, contractile vascular VSMC to cause transformation from a contractile to a synthetic phenotype with transmigration into the intima (580). Recent data suggest there may be multipotent vascular stem cells residing in the arterial media that are the actual cells that transmigrate.
and take on the VSMC phenotype once in the plaque (1755). Surprisingly, the well-studied cellular make-up of atherosclerotic lesions in animal models, including VSMC-like cells and various immune cells, has only recently been confirmed in human plaques (1933), demonstrating general comparability.

Promotion proceeds as retained and modified lipoproteins are taken up by macrophages and VSMC leading to formation of foam cells, the hallmark of the growing atherosclerotic lesion. While levels of LDL cholesterol (LDL-C), ApoB (apolipoprotein B), HDL-C (HDL cholesterol), or ApoA1 (apolipoprotein A1 or A-I) are clearly relevant, factors affecting plasma levels of these major risk factors will not be examined in this review. Recent studies with latex bead-labeled monocytes showed that changes in the rate of entry of monocytes into the plaque primarily determined changes in plaque size rather than impaired monocyte/macrophage egress. These studies were performed in ApoE-deficient mice before and after treatment with ApoE adenoviral vectors which rapidly normalized serum lipid levels and regressed plaques (1434).

As the lipid-rich plaque progresses, accumulating macrophages (and other migrating cells) as well as activated endothelial cells secrete a host of proinflammatory cytokines, matrix metalloproteinases (MMPs), and cathepsins, causing plaque fragility. IFN-γ strongly inhibits collagen formation by VSMC, further weakening the plaque and newly forming fibrous cap (1042). The result can be catastrophic thrombosis and downstream tissue infarction; but more often, there is limited mural thrombosis with subsequent organization of the thrombus, leading to saltatory growth of lesions with relatively high fibrous tissue content. Other precipitating changes in the plaque include erosions and, more especially, eruption through the endothelium of underlying cholesterol crystals (4). Prior to such episodes of thrombosis, there may be little, if any, encroachment of the plaque into the arterial lumen due to outward remodeling of the arterial wall to accommodate the growing plaque.

In reference to the tables that accompany the following sections, the percentage increase or decrease in atherosclerosis reported in the table represents differences in the extent of atherosclerosis in the genetically manipulated or targeted animals compared with control animals at the same point in time. The length of time of the experiments varies widely, and these times have only occasionally been noted in the tables. This limitation is balanced somewhat by utilizing atherosclerosis extent measured at the same point in time in the manipulated animals and their controls. As nearly all the studies cited were conducted in animals, mostly mice, relevance to the human condition may at times be questioned. Recent reviews regarding the relevance of animal models to humans note some limitations but emphasize the value of these models in providing general insights into shared common pathways (383, 609, 1300).

The measure of atherosclerosis generally utilized for this review is the quantitative plaque area measured at the aortic root if this measure was available. This site was chosen not because it is representative of the typical location of human lesions but because it is the most consistently quantified lesion location in mouse models and quantification at this location is relatively standardized. Indeed, humans essentially do not develop lesions in this location, except possibly patients with homozygous familial hypercholesterolemia. Aortic root atherosclerosis in mice is thought to be due to the unique hemodynamics of this animal model, possibly affected by the very rapid heart rate in mice. Nevertheless, these hemodynamic forces are generally thought to act on the endothelium in a way consistent with other hemodynamically predisposed areas. Assessment at multiple anatomic sites may provide a better assessment of disease involvement. Occasionally, discrepancies in disease responses are reported between the aortic root and other predisposed sites such as the aortic arch and brachiocephalic artery. No attempt has been made to consistently provide data on all these sites in this review, but a few exceptional differences in disease are pointed out in the tables.

Percent increase or decrease in atherosclerosis is admittedly a relatively crude measure of the effect size for a given manipulation. Nevertheless, there is a human counterpart. Plaque stability has gained much deserved attention as an extremely important factor in determining which specific plaque may cause an acute event. Indeed, most acute events, such as MI, appear to occur at sites of relatively modest sized plaques (494) or plaques poorly visualized by conventional angiography (44). However, these considerations should not overshadow the fact that accurate assessment of total plaque burden, as with coronary CT angiography, provides almost complete separation of future risk of coronary events ranging from near zero for no detectable plaque to extremely high risk for extensive plaque (689, 1281). Such scoring also predicts all-cause mortality (320). Even more crude assessment of plaque burden by coronary artery calcium scoring provides a strong gradient of risk (52, 224, 225, 1555).

III. FUNDAMENTAL SIGNALING PATHWAYS RELEVANT TO MULTIPLE STAGES OF ATHEROGENESIS

Some understanding of several basic signaling pathways, particularly in relation to inflammation in general, is a prerequisite to the study of atherogenesis. In this section, emphasis is given to endothelial signaling, in part because of the major role endothelial cells play in atherogenesis, and also because signaling transduction in endothelial cells is often neglected in reviews on atherogenesis. Nevertheless, the pathways presented in this section are so frequently encountered in multiple rele-
vant cell types (endothelial cells, smooth muscle cells, and leukocytes) that assignment to a specific stage of atherogenesis is difficult or entirely arbitrary.

A useful general overview of signaling pathways (136) begins with extracellular signals impinging on various cell receptors or sensors. These are linked to various intracellular signaling pathways through adaptor proteins or other transducers. The pathways utilize a host of signaling mechanisms including phosphorylation and other protein or membrane lipid modifications to transmit signals through amplifiers, filters, and multiple messengers to cellular effectors. Effectors may control vesicular transport for release of soluble signaling molecules such as cytokines or membrane expression of other cell signals (such as adhesion molecules), cell movement, contraction, phagocytosis, replication, or apoptosis. Frequently, the effectors are transcription factors that alter gene expression involved in all of the above functions. An attempt throughout this review will be made to maintain this outside-in orientation in the presentation of the various pathways.

A. Receptor Tyrosine Kinases

A surprisingly large number of signaling pathways occur on or in relation to cell membranes and involve alteration in membrane lipid components and/or translocation of signaling proteins to cell membranes. This principle is clearly demonstrated by the activity of receptor tyrosine kinases (RTK), illustrated in Figure 2 by insulin receptor (INSR) signaling. Impairment of insulin secretion as well as endothelial-specific KO of INSR increased atherosclerosis (see Table 1) as did knockout in other cells of the highly homologous insulin-like growth factor I receptor (IGF-IR). Further confirmation of the importance of these signaling pathways is seen in the multiple effects on atherosclerosis resulting from genetic manipulation of downstream molecules (Table 1). One major feature of INSR and other RTK is inhibition of apoptosis together with stimulation of growth and mitogenesis. Perhaps for this reason, insulin signaling may have quite opposite effects in endothelium compared with macrophages and other bone-marrow derived cells. Thus anti-apoptotic effects in endothelium seem to generally translate to protection against atherosclerosis based on endothelial-specific knockout of INSR and results from manipulation of downstream signaling molecules. On the other hand, impaired insulin signaling in macrophages often led to greater apoptosis, impaired proliferation, and smaller plaques in earlier stages, but potentially more complex and inflamed plaques at later stages of plaque development (see Table 1).

Insulin, IGF-I, PDGF, epidermal growth factor (EGF), VEGF vascular endothelial growth factor (VEGF), and many other growth factors signal via RTK. Indeed, without such signals, many cells in culture undergo spontaneous apoptosis. It is tempting to speculate that one of the anti-atherogenic aspects of INSR and IGF-IR signaling relate to inhibition of apoptosis. Other aspects of insulin signaling likely contribute. For example, insulin was also found to enhance endothelial cell-cell barrier function at adherens junctions, and this could be seen as anti-inflammatory.

An exception to the frequently anti-atherogenic effects of RTK signaling in endothelium appears to be fibroblast growth factor receptor 2 (FGFR2). While FGF family members normally promote angiogenesis, when a constitutively active form of FGFR2 was overexpressed in endothelium of ApoE-deficient mice, there was actually an overall antiproliferative and pro-apoptotic effect leading to increased atherosclerosis (288). The overall balance of the numerous pathways activated by RTK may therefore be critical for the outcome.

1. Tyrosine phosphorylation primarily provides docking sites

Tyrosine kinases in general and RTK in particular are evolutionarily the most recent of signaling innovations. Only 90 or so of the ~518 kinases in the human genome are tyrosine kinases, with most of the remaining being serine/threonine kinases (1036, 1137). The functions of tyrosine kinases are much more limited than the far more abundant and diverse serine/threonine kinases. The serine/threonine kinases include mitogen-activated protein kinase (MAPK), most of their activating upstream kinases as well as downstream kinases activated by MAPK, together with cAMP-dependent protein kinase (PKA), protein kinase B (PKB, also known as Akt), protein kinase C (PKC, a family with several isoforms), and cGMP-dependent protein kinase (PKG), calcium/calmodulin-dependent kinases (such as CaMKII), and a number of other kinase classes. In contrast, tyrosine kinases are divided into RTK (58 in 20 subfamilies) and non-RTK (32 in 10 subfamilies). Examples of non-RTK are the Src (short for sarcoma), JAK (Janus kinase), and Syk (spleen tyrosine kinase) families. Non-RTKs often work together with RTK or with other receptors that lack tyrosine kinase domains of their own. Both RTK and non-RTK typically activate downstream pathways by creating phosphorylated tyrosine residues that provide binding sites for assembly and activation of the next tier of signaling proteins. Molecules that bind phosphotyrosines possess requisite binding domains, such as phosphotyrosine binding (PTB) and Src-homology domain 2 (SH2) which recognize both the phosphorylated tyrosine and the surrounding peptide sequence.

INSR signaling is instructive in that all RTK share many of the same adapters and downstream signaling pathways. An exception may be the insulin receptor substrate (IRS) adapter proteins which are apparently restricted to insulin and IGF-I receptor signaling. The specificity of the particular receptor readout often comes more with the complete
FIGURE 2. Receptor tyrosine kinase signaling as illustrated by INSR (insulin receptor). Overall, cellular effects are dependent on simultaneous activation of multiple pathways. The multiplicity of activation pathways is achieved, in part, by binding of several different adaptor proteins (shown in amber) to either INSR or IRS1/2. Not shown are specific pathways leading to increased expression of GLUT4 by insulin (as occurs primarily in muscle and adipose). CCND1, cyclin D1; MTM1, myotubular myopathy 1, also known as myotubularin. See text for other abbreviations.
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<th>Gene</th>
<th>Model</th>
<th>%Δ A</th>
<th>Function, Comment</th>
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<tr>
<td><strong>INS2</strong></td>
<td>INS2&lt;sup&gt;−/−&lt;/sup&gt; Akita in LDLR&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑ 224% males, ↑ 30% females</td>
<td>The INS2 (insulin 2) Akita mutation (Cys96Tyr) causes misfolding of insulin, beta-cell ER stress and apoptosis, and in heterozygotes, type I DM by 4–5 wk of age. Serum lipids were increased markedly as well compared to LDLR null controls. Arterial wall expression of MCP-1, TNF-α, and IL-1β were also increased (2102), but the contribution of insulin deficiency itself is unclear.</td>
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<td>INS2&lt;sup&gt;−/−&lt;/sup&gt; Akita in apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑ 211%</td>
<td>As in LDLR null mice, apoE-deficient Akita mice had marked increases in serum cholesterol, complicating the interpretation. Interestingly, there was no increase in VLDL production and little increase in serum triglycerides but elevated APOB-48 in the LDL density range (867).</td>
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<td><strong>IGF-I</strong></td>
<td>Congenic 6T allele in apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑ 88%</td>
<td>The congenic 6T allele resulted in mild (41%) reduction in IGF-I. The increase in atherosclerosis was on normal diet in the aortic root but the difference was not significant on western diet while overall aortic area remained increased. There may have been differences in other, unknown genes in the mice with the congenic 6T alleles (1599).</td>
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<td><strong>PAPP-A</strong></td>
<td>PAPP-A&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↓ 70%</td>
<td>PAPP-A (pregnancy-associated plasma protein A) is a metalloproteinase in the IGF system and leads to increased lesional availability of IGF-I and increased VSMC proliferation (722).</td>
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<td>SMC-specific Tg PAPP-A&lt;sup&gt;−/−&lt;/sup&gt; in apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑ 250%</td>
<td>Overexpression increased atherosclerosis (350).</td>
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<td><strong>INSR</strong></td>
<td>ESMIRO apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑ 52%</td>
<td>ESMIRO (endothelial-specific INSR knockout) effect may be due to lack of normal insulin stimulation of eNOS (576). Approximately threefold greater lesion volume was seen in another study (1458).</td>
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<td>INSR&lt;sup&gt;−/−&lt;/sup&gt; BMT into LDLR&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑ 45%</td>
<td>Lesions had larger necrotic cores. INSR&lt;sup&gt;−/−&lt;/sup&gt; macrophages had decreased PKB/Akt signaling and increased ER stress and susceptibility to apoptosis (707).</td>
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<td>MphIRKO in apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↓ 50%</td>
<td>Macrophage-specific knockout (Cre-lox) of INSR (116).</td>
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<td><strong>IGF-IR</strong></td>
<td>Macrophage-specific IGF-IR&lt;sup&gt;−/−&lt;/sup&gt; in apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑ 57%</td>
<td>This model resulted in an increase in macrophage infiltration while VSMC content decreased together with collagen resulting in fibrous cap disruption and intraplaque hemorrhage (762). Included here with other IGF-related genes though not directly related to VSMC IGF signaling.</td>
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<td><strong>FGFR2</strong></td>
<td>Endothelial cell-specific Tg of constitutively active FGFR2 in apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑ 69%</td>
<td>FGFR2 (fibroblast growth factor receptor 2) is also involved in angiogenesis. Given here is the effect at the aortic sinus. Increased expression of p21&lt;sup&gt;Cip1&lt;/sup&gt;, a cell-cycle inhibitor, also increased adhesion molecules, PDGF and the transcription factor Egr-1 were also increased (288).</td>
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<tr>
<td><strong>IRS1</strong></td>
<td>INSR&lt;sup&gt;−/−&lt;/sup&gt; IRS1&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑ 99%</td>
<td>Shown are effects in aorta of female mice. Despite much higher plasma insulin, eNOS phosphorylation and endothelial-dependent vasodilatation was reduced in these double heterozygous mice. Oddly, baseline ERK1/2 activation was reduced, but insulin-stimulated ERK1/2 activation was increased in aortic wall homogenates. VCAM-1 expression was increased. BMT studies showed vascular wall effects predominated (582).</td>
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Table 1.—Continued

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<tr>
<td>IRS2</td>
<td>IRS2/– apoE/–</td>
<td>↑ 60%</td>
<td>Atherosclerosis difference in aortic root given. Whole body IRS2/– mice had increased atherosclerosis while adoptive transfer of IRS2/– fetal liver cells (FLC) into irradiated apoE/– mice led to less atherosclerosis. In response to LPS, IRS2 KO macrophages increased TNF-α and MCP-1 normally but production of IL-1β and IL-6 were completely abrogated (116). Increase in atherosclerosis correlated with plasma insulin levels in another study of whole-body IRS2/– (637).</td>
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<td>FLC IRS2/– into apoE/–</td>
<td>↓ 38%</td>
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<td>IRS2 KO macrophages increased TNF-α and MCP-1 normally but production of IL-1β and IL-6 were completely abrogated. Increase in atherosclerosis correlated with plasma insulin levels in another study of whole-body IRS2/– (637).</td>
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<td>HDAC3</td>
<td>shHDAC3 apoE/–</td>
<td>↑ 280%</td>
<td>Heterozygous deficiency modestly increased atherosclerosis only on atherogenic diet. Associated with increased macrophage uptake of acetylated LDL and higher expression of CD36, SRA, and MCP-1. Pharmacological inhibition of Akt had similar effects (638).</td>
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<td>FOXO4</td>
<td>EC-specific FOXO4/– apoE/–</td>
<td>↑ 42%</td>
<td>Mice were exposed to infused ANG II as well. Percent increase in atherosclerosis was not reported in abstract (765, 766).</td>
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<td>PI3Kγ (PIK3CG)</td>
<td>p110γ/– apoE/–</td>
<td>↓ 52%</td>
<td>Also known as PI3KG (phosphatidylinositol 3-kinase, catalytic, gamma or the p110 gamma catalytic subunit). PI3Kγ isoform knockout not embryo lethal (others are) (277). While having pro-survival effects with PKB activation, expression of endothelial E-selectin appears to be dependent on PI3Kγ (1441). PI3Kγ also regulates chemotaxis as PI3Kγ is activated by βγ subunits of GPCR by various chemokines and is required for neutrophil and other leukocyte migration. Assessed at 35 wk in the aortic root. Less reduction at later time points. BMT into LDL KO mice suggest macrophages and T-cells effects explain much of the atherosclerosis reduction (534). Macrophage PI3Kγ is activated by oxLDL, inflammatory cytokines, and ANG II.</td>
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<td>Akt1</td>
<td>Akt1/– apoE/–</td>
<td>↑ 52%</td>
<td>Also known as PKBα. Akt1 is the main endothelial isoform. Akt phosphorylates and activates eNOS. BMT of Akt1-null macrophages did not increase atherosclerosis. Extensive coronary atherosclerosis seen (519). Impaired VSMC function, vulnerable plaques result in acute MI (520).</td>
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<tr>
<td>FOXO1</td>
<td>FOXO1KR/KR LDLR/–</td>
<td>↑ 39%</td>
<td>Knock in of the KR allele of FOXO1 results constitutive deacetylation and preferential accumulation in the nucleus (thus, KR is a gain of function mutation). Insulin favors FOXO1 acetylation while SIRT1 deacetylates FOXO1. Insulin, through Akt1, also increases FOXO1 phosphorylation and exclusion from the nucleus (1442). Cons</td>
</tr>
<tr>
<td>FOXO3</td>
<td>EC-specific FOXO3/–, FOXO3/–, and FOXO4/– in LDLR/–</td>
<td>↓ 77%</td>
<td>The large effect from the EC-specific KO of all FOXOs suggest that FOXO3 has an additional pro-atherosclerotic effect in EC beyond that of FOXO1 alone (1817). FOXO3 transcription factors in EC increase expression of IRS1 and IRS2 but strongly suppress expression of eNOS while increasing iNOS. Remarkably, the same KO model restricted to myeloid cells resulted in a nearly twofold increase in atherosclerosis with proliferation of myeloid cells and generation of increase RNS through iNOS (1818).</td>
</tr>
<tr>
<td>FOXO4</td>
<td>FOXO4/– apoE/–</td>
<td>↑ 128%</td>
<td>FoxO4 null BMT also increased atherosclerosis (in C57Bl/6 mice). FOXO4 is a transcription factor that in macrophages can inhibit several aspects of NF-κB signaling including IL-6 production and transcription of ROS-scavenging enzymes. Macrophage ROS and serum IL-6 were increased in FoxO4 null mice (2111).</td>
</tr>
<tr>
<td>HDAC3</td>
<td>shHDAC3 apoE/–</td>
<td>↑ 280%</td>
<td>HDAC3 (histone deacetylase 3) is phosphorylated by Akt/PKB in areas of disturbed flow and promotes endothelial cell survival. Model shown here involved lentivirus infection and knockdown in the endothelial monolayer of aortic isografts transplanted to carotid arteries. Knockout is embryo-lethal (2057).</td>
</tr>
<tr>
<td>SIRT1</td>
<td>SIRT1/– apoE/–</td>
<td>↑ 112%</td>
<td>SIRT1 (sirtuin 1) is also a deacetylase. BMT of SIRT1– cells resulted in a 54% increase in atherosclerosis, suggesting approximately equal contributions from endothelial and macrophage effects. SIRT1– macrophages took up oxLDL more avidly (1680).</td>
</tr>
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</table>
**MOLECULAR BIOLOGY OF ATHEROSCLEROSIS**

**Table 1.—Continued**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Model</th>
<th>%Δ A</th>
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<tbody>
<tr>
<td>PKCβ</td>
<td>PKCβ^−/− apoE^−/−</td>
<td>↓ 75%</td>
<td>Disturbed flow and oxLDL can lead to activation of PKCβ, activation of JNK, and Egr-1, all blocked in PKCβ^−/− mice (720). Inhibition of PKCβ by inhibitor or siRNA in macrophages also prevents foam cell formation, possibly by reducing scavenger receptor A expression (1355). PKCβ^−/− mice are also immunodeficient.</td>
</tr>
<tr>
<td>PKCβ3</td>
<td>PKCβ3^−/− apoE^−/−</td>
<td>↓ 50%</td>
<td>17 wk, aortic root (1925).</td>
</tr>
<tr>
<td>p47phox</td>
<td>p47phox^−/− apoE^−/−</td>
<td>↓ 70%</td>
<td>Also known as Ncf1 (neutrophil cytosolic factor 1). &quot;Organizer&quot; subunit for NOX2 complex assembly and function. p47phox^−/− mice are also a model of autosomal chronic granulomatous disease. Total aortic plaque surface area decreased as shown, but no decrease was seen in aortic sinus plaque (113). Bone marrow-derived and vascular wall cells appear to show equal contributions (1872). Also required for NF-κB signaling by TNFα (and others) (1026). Upregulation of p47phox may mitigate the effects of p47phox knockout (496).</td>
</tr>
<tr>
<td>NOX2 (gp91phox)</td>
<td>NOX2^+/− apoE^−/−</td>
<td>↓ 48%</td>
<td>NOX2 (NADPH oxidase 2) was clearly present in endothelial cells and macrophages. Deficiency causes X-linked chronic granulomatous disease (CGD). Given is plaque area reduction in aorta (866). A prior study (914) found only a trend in total lesion area reduction in male C57 Cd mice and no effect in males or females at the aortic sinus at 24 wk.</td>
</tr>
<tr>
<td>NOX1</td>
<td>NOX1^−/− apoE^−/−</td>
<td>↓ 28%</td>
<td>NOX1 (NADPH oxidase 1) is primarily expressed in VSMC. Shown is percent reduction at the aortic sinus. Carotid ligation promotes VSMC proliferation which was inhibited by NOX1 deficiency as well (1609).</td>
</tr>
<tr>
<td>PTEN</td>
<td>PTEN^+/− apoE^−/−</td>
<td>NS</td>
<td>Whole body heterozygous deletion of PTEN decreased aortic PTEN about 50% and increased Akt/PKB activity 50% but had no effect on atherosclerosis (64).</td>
</tr>
<tr>
<td>G6PDH</td>
<td>G6PDH^+/− apoE^−/−</td>
<td>↓ 43%</td>
<td>G6PDH (glucose-6-phosphate dehydrogenase) is a key (X-linked) enzyme for the pentose phosphate pathway and production of NADPH. The mutation caused 80% reduction of G6PDH activity and 50% reduction NADPH in the aorta of male hemizygous mice together with a marked reduction in superoxide production by NOX (1155).</td>
</tr>
<tr>
<td>AMPKα2</td>
<td>AMPKα2^+/− LDLR^−/−</td>
<td>↑ 48%</td>
<td>AMPK is increased by laminar flow. It phosphorylates and activates eNOS, decreases superoxide, and suppresses ER stress. The α2 subunit was most active in this regard (435). Also important in macrophage ER stress.</td>
</tr>
<tr>
<td><strong>MAPK-related pathways</strong></td>
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<tr>
<td>Grb2</td>
<td>Grb2^+/− apoE^−/− and Grb2^−/− BMT</td>
<td>↓ 43%</td>
<td>Grb2^−/− is embryolethal. Grb2 is an adapter protein that couples activated (tyrosine phosphorylated) growth factor receptors, integrins, or other adapters to the MAPK pathway through SOS. Similar whole-body and BMT results suggest most of effect is due to effects on hematopoetic cells. MAPK activation is required for macrophage proliferation and uptake of modified lipoproteins (1439).</td>
</tr>
<tr>
<td>ASK1</td>
<td>ASK1^−/− apoE^−/−</td>
<td>↑ 131%</td>
<td>ASK1-null mice showed less macrophage apoptosis resulting in larger lesions and greater MMP expression but lesions showed less necrosis. Similar results with ASK1^−/− BMT (2005).</td>
</tr>
<tr>
<td>AIP1</td>
<td>AIP1^−/− apoE^−/−</td>
<td>↑ 86%</td>
<td>An anti-inflammatory effect was seen primarily in endothelial cells (793).</td>
</tr>
<tr>
<td>p38</td>
<td>macrophage-specific p38 deletion (Cre-lox) in apoE^−/− mice</td>
<td>↑ 14% (NS)</td>
<td>p38 is a MAPK. While the increase in lesion size was not significant, there was certainly no decrease as might have been expected. Instead, there was marked increase in apoptotic markers, necrotic areas, a decrease in collagen deposition, and thinning of the fibrous cap (1583). Uptake of apoptotic cells by macrophages (efferocytosis) triggers an anti-inflammatory release of IL-10 and TGFβ dependent on p38 (473).</td>
</tr>
<tr>
<td>JNK1 (MAPK8)</td>
<td>JNK1^−/− apoE^−/−</td>
<td>NS</td>
<td>JNK2 but not JNK1 KO decreased atherosclerosis (1483).</td>
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<table>
<thead>
<tr>
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<tr>
<td>JNK2 (MAPK9)</td>
<td>JNK2&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↓53%</td>
<td>In JNK2 KO, macrophage SR-A was not phosphorylated and activity was decreased with less foam cell formation. Other aspects of inflammation are likely also affected (1483).</td>
</tr>
<tr>
<td>Egr1</td>
<td>Egr1&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↓86%</td>
<td>Lesions were also less complex. Egr1 is a transcription factor activated by the MAPKs ERK1/2 and JNK. Egr1&lt;sup&gt;−/−&lt;/sup&gt; aorta showed reduced MCP-1, ICAM-1, and VCAM-1 expression. oxLDL activates Egr1 in macrophages as well (719). Egr-1 deficiency in bone marrow-derived cells reduced atherosclerosis 28%, suggesting both endothelial and macrophage effects. Egr-1 deficient macrophages expressed less VCAM-1 and tissue factor which are Egr-1 target genes (43).</td>
</tr>
<tr>
<td>MK2</td>
<td>MK2&lt;sup&gt;−/−&lt;/sup&gt; LDLR&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↓56%</td>
<td>Both endothelial activation and macrophage foam cell formation were affected (840). MK2 is a target of p38. It can phosphorylate p47phox to activate NOX2 in angiotensin II signaling (458). Also known as MAPKAPK2 (MAPK activated protein kinase 2).</td>
</tr>
<tr>
<td>MKP-1</td>
<td>MKP-1&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↓50%</td>
<td>MKP-1 (MAPK phosphatase 1) inactivates several MAPKs. The result seems paradoxical (1612). Confirmed by second study (820). Both groups concurred that the atherosclerosis protection was due to macrophage effects with less MCP-1 produced by MKP-1&lt;sup&gt;−/−&lt;/sup&gt; macrophages and less influx of inflammatory macrophages into lesions. Transplant of wild type bone marrow into MKP-1&lt;sup&gt;−/−&lt;/sup&gt; mice restored susceptibility to atherosclerosis (1612).</td>
</tr>
<tr>
<td>NR4A1</td>
<td>NR4A1&lt;sup&gt;−/−&lt;/sup&gt; BMT in apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑114%</td>
<td>NR4A1 (nuclear receptor 4A1, also known as Nur77) is an orphan nuclear receptor phosphorylated by Erk5 that may act as a transrepressor of NF-κB signaling by binding p65. NR4A1-deficient macrophages displayed greater classical (M1) activation with greater TLR4 expression and more iNOS, IL-12, IFNγ, and much greater CXCL12, but less IL-10 expression in response to LPS (702).</td>
</tr>
<tr>
<td>ADAM17</td>
<td>ADAM17&lt;sup&gt;FVB/FVB&lt;/sup&gt; LDLR&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↓50%</td>
<td>TACE (TNFα cleavage enzyme)/ADAM17 cleaves TNFα, TNFR1, ICAM-1, VCAM-1, and CX3CL1 from the external side of their membrane origins. ADAM17 also promotes shedding of several EGFR ligands. Atherosclerosis-resistant FVB backcrossed with susceptible C57BL/6J in LDLR&lt;sup&gt;−/−&lt;/sup&gt; background showed less atherosclerosis linked to increased ADAM17 expression and activity in FVB mice due to a −25T&gt;G promoter variant (777).</td>
</tr>
<tr>
<td>TNFα</td>
<td>TNFα&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↓60%</td>
<td>Produced in EC (and other cells) in response to NF-κB signaling. Produced by monocytes and macrophages in response to IFN-γ. Also secreted by mast cells. Strong activator of endothelial cells. TNFα KO had a lesser or negative effect in some studies (918, 919). TNFα knockout in atherogenic diet-fed C57BL/6J did not result in a decrease in atherosclerosis (1565). Similar reduction (69%) as shown here at aortic root seen at 6 wk in TNFα&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt; in another study (1991). Only 27% reduction at 12 wk with greater endothelial ICAM-1, VCAM-1, MCP-1 expression and increased oxLDL uptake in macrophages in TNFα KO mice in another (1333). Differences in mouse lesion progression detectable by MRI (926).</td>
</tr>
<tr>
<td>LTα</td>
<td>LTα&lt;sup&gt;−/−&lt;/sup&gt; C57BL/6</td>
<td>↓62%</td>
<td>Lymphotoxin-α (also known as TNF-β) is a soluble cytokine in the TNF family. Ligates both TNFR1 and TNFR2. Its presence in atherosclerotic lesions was also confirmed (1565).</td>
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Table 1.—Continued

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<tbody>
<tr>
<td>TNFR1</td>
<td>TNFR1&lt;sup&gt;+/−&lt;/sup&gt; atherogenic diet fed C57BL/6J mice</td>
<td>↑130%</td>
<td>TNF-α receptor 1 (p55). Controversial results (918, 919, 1565). Artery segments from wild type mice expressed more TNFR1 with age and developed atherosclerosis more rapidly after transplant.</td>
</tr>
<tr>
<td></td>
<td>TNFR1&lt;sup&gt;−/−&lt;/sup&gt; carotid arteries transplanted into apoE&lt;sup&gt;−/−&lt;/sup&gt; mice</td>
<td>↓70%</td>
<td>Age did not affect atherosclerosis in TNFR1 deficient transplants (2078). Shown is difference in intima areas. Lesser differences were seen in young transplanted segments. Correlative data on human variants given as well.</td>
</tr>
<tr>
<td>TNFR2</td>
<td>TNFR2&lt;sup&gt;+/−&lt;/sup&gt; atherogenic diet fed C57BL/6J mice</td>
<td>NS</td>
<td>The TNFR2 (p75) receptor lacks death domains and can be anti-apoptotic but can also generate active NIK (1565). TNFR2 activates the alternative NF-κB pathway. TNFR1 does not. TNFR2 only responds to membrane-bound TNF and upregulates membrane TNF expression, thereby also increasing TNFR1 signaling. TNFR2 can promote apoptosis by recruitment of TRAF2 and cIAP1/2 from TNFR1 (1459).</td>
</tr>
<tr>
<td></td>
<td>TNFR2&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt; mice</td>
<td>↓43%</td>
<td>TNFR2&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt; mice had twofold elevations in plasma TNF-α and IL-17 (possibly inflammatory) and IL-10 (anti-inflammatory). TNFR2 also mediated much of the TNF-α induced E-selectin, ICAM-1, and VCAM-1 expression in EC as well as leukocyte capture. TNFR1 and TNFR2 had different but overlapping effects in EC (276).</td>
</tr>
<tr>
<td>TRAF1</td>
<td>TRAF1&lt;sup&gt;−/−&lt;/sup&gt; LDLR&lt;sup&gt;−/−&lt;/sup&gt; mice</td>
<td>↓32%</td>
<td>Plaques showed marked reduction in macrophages. BMT studies suggested roughly equal contributions to reduction in atherosclerosis from endothelial and macrophage effects. Reduced VCAM-1, ICAM-1 and leukocyte adhesion in endothelial cells. Decreased monocyte expression of integrin β1 and adhesion to activated endothelial cells. TRAF1&lt;sup&gt;−/−&lt;/sup&gt; mice had reduced serum and tissue levels of IL-6 and TNF-α (1217).</td>
</tr>
<tr>
<td>TRAF5</td>
<td>TRAF5&lt;sup&gt;−/−&lt;/sup&gt; LDLR&lt;sup&gt;−/−&lt;/sup&gt; mice</td>
<td>↑44%</td>
<td>Plaques had more macrophages. TRAF5&lt;sup&gt;−/−&lt;/sup&gt; endothelial cells expressed more VCAM-1 and ICAM1. TRAF5&lt;sup&gt;−/−&lt;/sup&gt; monocytes expressed more a4 integrin (CD49d) and greater acetyl-LDL uptake. There was greater MCP-1 expression and JNK activation upon stimulation by TNF-α (1218).</td>
</tr>
<tr>
<td>NEMO</td>
<td>endothelial-specific Cre-lox NEMO ablation in apoE&lt;sup&gt;−/−&lt;/sup&gt; mice</td>
<td>↓30–47%</td>
<td>Two endothelial NEMO ablation models were tested. NEMO ablation blocked NF-κB signaling. Less advanced lesions seen (592).</td>
</tr>
<tr>
<td>IKK2</td>
<td>Liver-specific IKK2 Tg in apoE-Leiden mice</td>
<td>↑131%</td>
<td>Also known as IKKB (inhibitor of kappa light chain gene enhancer in B cells, kinase of, beta). The marked increase in atherosclerosis was associated with only mild or minimal evidence of additional systemic inflammation and there were temporarily mild increases in LDL and VLDL-C (1975).</td>
</tr>
<tr>
<td></td>
<td>Macrophage-specific IKK2 deletion (Cre-lox) in LDLR&lt;sup&gt;−/−&lt;/sup&gt; mice</td>
<td>↑62%</td>
<td>There was only 65% inhibition of IKK2 expression in macrophages seen with 50% reduction in NF-κB activation. Lesions were more advanced and displayed greater necrosis. Macrophages produced more TNF-α upon stimulation and less anti-inflammatory IL-10 (881).</td>
</tr>
<tr>
<td>IkBα</td>
<td>Endothelial-specific degradation-resistant IkBα in apoE&lt;sup&gt;−/−&lt;/sup&gt; mice</td>
<td>↓60%</td>
<td>Percent reduction in aortic sinus given. Marked reduction seen in NF-κB signaling with decreased adhesion molecule and cytokine expression as well as reduced macrophage and T-cell entry into plaques (592).</td>
</tr>
<tr>
<td></td>
<td>IkBα&lt;sup&gt;−/−&lt;/sup&gt; Cre-lox BMT in apoE&lt;sup&gt;−/−&lt;/sup&gt; mice</td>
<td>↑90%</td>
<td>IkBα&lt;sup&gt;−/−&lt;/sup&gt; macrophages secreted more CCL5 and displayed increased adhesion to endothelial cells (640).</td>
</tr>
<tr>
<td>cIAP2</td>
<td>cIAP2&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt; mice</td>
<td>↓47%</td>
<td>cIAP2 and cIAP1 are E3 ubiquitin ligases capable of synthesizing either K48 or K63 chains and are involved in both activation (TNFR1 signaling with RIP1 K63 target, CD40 with K48 TRAF3 target, or TNFR2 signaling with K48 TRAF2 target) or inhibition (K48 NIK target) of NF-κB signaling. See Table 2. These results suggest net effect of cIAP2 is proinflammatory (1643).</td>
</tr>
<tr>
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<tr>
<td>NF-κB1</td>
<td>NF-κB1−/− BM into LDLR−/−</td>
<td>↓ 41%</td>
<td>NF-κB1 encodes the p50 subunit—one of the components that can make up dimeric NF-κB. Though atherosclerotic lesion size was decreased the lesions had more inflammatory cells but near absence of foam cells. There was decreased uptake of oxidized LDL and reduced expression of SR-A (880). (Entry placed here to keep NF-κB signalosome together though effect is predominantly on macrophages and could be considered a promoter.)</td>
</tr>
<tr>
<td>NR4A3</td>
<td>NR4A3−/− apoE−/−</td>
<td>↓ 52%</td>
<td>NR4A3 (nuclear receptor 4A3, also known as NDR1) was induced by NF-κB and transactivated VCAM-1 and ICAM-1 expression in endothelial cells. Fewer macrophages were seen in lesions of KO animals (2096).</td>
</tr>
<tr>
<td>A2O (TNFAIP3)</td>
<td>A2O+− apoE−/−</td>
<td>↑ 60%</td>
<td>Shown is effect in males. Only 23% increase was seen in females (1971). Decreased atherosclerosis was seen with A2O over-expression. A2O inhibits TRAF-mediated activation of NF-κB signaling (TRAF2 for TNFR1, TRAF6 for IL-1R and TLR4) by degrading K63 ubiquitin chains.</td>
</tr>
<tr>
<td>GR</td>
<td>BMT of macrophage-specific GR−/− into LDLR−/−</td>
<td>NS</td>
<td>GR (glucocorticoid receptor) deficiency in macrophages did not affect lesion size but did decrease lesion calcification (1437).</td>
</tr>
<tr>
<td>SCD1</td>
<td>SCD1−/− LDLR−/−</td>
<td>↑ 78%</td>
<td>Mice deficient in SCD1 (steroyl-CoA desaturase 1) had more atherosclerosis despite less weight gain, lower triglycerides and greater insulin sensitivity. 78% increase represents the average of male and female in 2 separate SCD1 deficiency models. The mice showed extensive skin inflammation as well. BMT of SCD1-deficient cells did not affect lesions. (1105). SCD1 may be important for preventing the production of highly inflammatory ceramide from palmitate by converting palmitate to palmitoleic acid instead or providing unsaturated fatty acids for phosphatidyl choline synthesis to alleviate ER stress (484, 655, 760, 1410).</td>
</tr>
<tr>
<td>LYPLA3 (PLA2G15)</td>
<td>LYPLA3−/− apoE−/−</td>
<td>↑ 78%</td>
<td>LYPLA3 (lysophospholipase 3) is also known as LLPL (LCAT-like lysophospholipase), O-acyl-ceramide synthase (ACS), or PLA2G15. Protective effect of normal activity thought due to acylation of ceramide. Macrophages from LYPLA3 KO mice were more prone to apoptosis upon exposure to oxLDL (1758).</td>
</tr>
<tr>
<td>GLA</td>
<td>GLA−/− apoE−/−</td>
<td>↑ 104%</td>
<td>Deficiency of the X-linked GLA (α-galactosidase A) causes Fabry disease. Membrane-associated increases in globotriaosylceramide. GLA deficient mice in this study had greatly increased aortic iNOS expression and increased nitrotyrosine (162). Alterations in endothelial caveolar signaling may be involved with eNOS inhibition (1629).</td>
</tr>
<tr>
<td>IL-1Ra</td>
<td>IL-1Ra Tg LDLR−/−</td>
<td>↓ 40%</td>
<td>IL-1 receptor antagonist. A naturally occuring antagonist of IL-1 action. Knockout leads to massive inflammation (918).</td>
</tr>
<tr>
<td>IL-1α</td>
<td>IL-1α−/− C57BL/6</td>
<td>↓ 56%</td>
<td>Increases endothelial activation, elaboration of adhesion molecules. Induces production of broad range of cytokines/chemokines. Also produced by activated EC, VSMC, and T-cells (918).</td>
</tr>
<tr>
<td>IL-1β</td>
<td>IL-1β−/− apoE−/−</td>
<td>↓ 33%</td>
<td>Similar effects as IL-1α (918). Recent studies show IL-1 production by macrophages and IL-1R on endothelial cells are main determinants of IL-1 mediated atherosclerosis (875).</td>
</tr>
<tr>
<td>IL-1R1</td>
<td>IL-1R1−/− apoE−/+</td>
<td>↓ 93%</td>
<td>IL-1 receptor 1. Included gingivitis in model to promote atherosclerosis (918). Most important site is EC (875, 1610). Less marked effects reported previously without gingivitis (270). Plaques in IL-1R1 null apo E deficient mice reported to have fewer VSMC, less collagen, more hemorrhage, and less outward remodeling in more distal vessels (such as brachiocephalic artery), with reduced expression of MMP3 (47).</td>
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-Ceramide metabolism (relates to TNFR1 activity)-

-IL-1Ra signaling including activation by IL-1β and other TNF superfamily receptors
**Table 1.**—Continued

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<tr>
<td>OPG (TNFRSF11B)</td>
<td>OPG&lt;sup-&gt;&lt;/sup&gt;/apoE&lt;sup-&gt;&lt;/sup&gt;</td>
<td>↑27%</td>
<td>OPG (osteoprotegerin), also known as TNFRSF11B (tumor necrosis factor receptor superfamily, member 11B) acts as a decoy receptor for RANKL (receptor activator of NF-κB ligand) and thus inhibits signaling through the RANK receptor. Shown is the mean effect of male and female mice at 60 wk (125).</td>
</tr>
<tr>
<td>AT1R</td>
<td>AT1aR&lt;sup-&gt;&lt;/sup&gt;/apoE&lt;sup-&gt;&lt;/sup&gt;</td>
<td>↓54%</td>
<td>Given here is the effect of whole body knockout. Decreased plaque rupture seen in another model (68). Macrophage-specific AT1aR deficiency had only a small effect (563) or even increase atherosclerosis (889) but a clear reduction in atherosclerosis was seen with AT1R null BMT when accompanied by ANG II-stress from uninephrectomy (2008). Reduced plaque (by 86%) seen also with matched blood pressure (384, 1929).</td>
</tr>
<tr>
<td>AT2R</td>
<td>AT2R&lt;sup-&gt;&lt;/sup&gt;/apoE&lt;sup-&gt;&lt;/sup&gt; with diabetes</td>
<td>↓23%</td>
<td>The apparent proatherosclerotic influence of intact AT2R was seen with pharmacologic blockade as well (930). An earlier study had found no effect in LDLR deficient mice (384).</td>
</tr>
<tr>
<td>ROCK1</td>
<td>ROCK1&lt;sup-&gt;&lt;/sup&gt;/apoE&lt;sup-&gt;&lt;/sup&gt;</td>
<td>↓60%</td>
<td>ROCK1/2 as effectors of RhoA are involved in many inflammatory effects as well as smooth muscle contraction. Involved in leukocyte movement control through RhoA signaling (1835).</td>
</tr>
<tr>
<td>ROCK2</td>
<td>ROCK2&lt;sup-&gt;&lt;/sup&gt;/apoE&lt;sup-&gt;&lt;/sup&gt;</td>
<td>↓45%</td>
<td>ROCK2 KO macrophages showed increased PPARγ and LXRα activation with greater expression of ABCA1 and cholesterol efflux (2104).</td>
</tr>
<tr>
<td>PYK2</td>
<td>PYK2&lt;sup-&gt;&lt;/sup&gt;/apoE&lt;sup-&gt;&lt;/sup&gt;</td>
<td>↓66%</td>
<td>Additional studies with BMT showed that arterial wall and bone marrow-derived cells contributed similarly to the reduction in atherosclerosis (890).</td>
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**Inflammatory GPCR signaling as exemplified by AT1R and the renin-angiotensin system**

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<th>Gene</th>
<th>Model Description</th>
<th>%ΔA</th>
<th>Function, Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGT</td>
<td>human AGT/renin Tg C57BL/6J mouse</td>
<td>↑300%</td>
<td>Compared with C57BL/6J both fed atherogenic diet. Effects on blood pressure were not controlled for (1697).</td>
</tr>
<tr>
<td>Renin</td>
<td>renin&lt;sup/-/-&lt;/sup&gt; BMT in LDLR&lt;sup/-/-&lt;/sup&gt;</td>
<td>↓66%</td>
<td>Though renin deficiency in bone marrow-derived cells decreased atherosclerosis, the major pro-atherosclerotic effect of ANG II appears to be through AT1R on endothelial cells (1080).</td>
</tr>
<tr>
<td>ACE</td>
<td>“soluble ACE” apoE&lt;sup-&gt;&lt;/sup&gt;</td>
<td>↓43%</td>
<td>Mice homozygous for the soluble ACE mutation had an 86% reduction in plasma ACE activity as well as reduced blood pressure. The reduction in atherosclerosis was shown to be dependent on the blood pressure (and presumable total body ANG II production) rather than the specific location of the ACE (1938). Similar results in heterozygous ACE deficiency in apo E null mice (737).</td>
</tr>
<tr>
<td>ACE2</td>
<td>Tg ACE2 in high fat fed rabbits with arterial injury</td>
<td>↓58%</td>
<td>Shown is reduction in intimal area at the aortic root. ACE2 cleaves ANG I into inactive ANG 1-9 and ANG II into vasodilating ANG 1-7, thereby opposing the activity of ACE (2066). ACE2 is on the X chromosome.</td>
</tr>
<tr>
<td>ACE2&lt;sup-&gt;&lt;/sup&gt;/apoE&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td></td>
<td>↑325%</td>
<td>Increased expression of several inflammatory markers on endothelial cells was seen and increased atherosclerosis was inhibited by perindopril (an ACE inhibitor) (1786).</td>
</tr>
<tr>
<td>ACE2&lt;sup-&gt;&lt;/sup&gt;/apoE&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td></td>
<td>↑38%</td>
<td>Increased atherosclerosis in both whole body and BMT studies. ACE2 in lesions associated with macrophages. ACE2 deficient macrophages secreted more ANG II and co-infusion of ANG 1-7 blocked excess atherosclerosis (1781).</td>
</tr>
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Continued
Table 1.—Continued

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<thead>
<tr>
<th>Gene</th>
<th>Model</th>
<th>%ΔA</th>
<th>Function, Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>p21Cip1 (CDKN1A)</td>
<td>p21Cip1−/− apoe−/−</td>
<td>↓53%</td>
<td>Increased expression of cyclin-dependent kinase inhibitor p21Cip1 was, in part, dependent on PYK2 and associated JNK signaling and acted as a co-activator for VCAM-1 and MGP-1 transcription in endothelial cells (890). Macrophage apoptosis was increased while macrophages showed less inflammation and greater efferocytosis capability with p21Cip1 KO. Fibrous caps were thicker. Results confirmed with different diets and BMT studies (1196). Similar results (34% reduction in atherosclerosis) was seen in apo E null mice with ANGII infusion which increased p21Cip1 expression (961).</td>
</tr>
<tr>
<td>STAT3</td>
<td>endothelial-specific STAT3−/− C57BL/6 mice fed an atherogenic diet</td>
<td>↓64%</td>
<td>In this study, endothelial JAK2/STAT3 was activated by oxidized phospholipid (610). JAK2 activation was dependent on Src but likely also on PYK2 with signaling through CD36 (716). In another study, increased expression of STAT3 by adenovirus delivery of human STAT3 appeared protective with a 15% reduction in atherosclerosis. Treg-associated Foxp3 and SOCS1 expression were increased while macrophage burden was decreased (901). Dual delivery of both STAT3 and IL-10 were not additive in their anti-atherosclerotic effects (252).</td>
</tr>
<tr>
<td>GRK2</td>
<td>GRK2−/− BMT in LDLR−/−</td>
<td>↓31%</td>
<td>Complete GRK2 (G-protein coupled receptor kinase-2) KO is embryo lethal. Macrophage-specific GRK2 KO did not affect atherosclerosis (1359). GRK2 phosphorylation may mark GPCR for chemokines or AT1R for β-arrestin2 with inflammatory coupling.</td>
</tr>
<tr>
<td>APLN</td>
<td>APLN−/− apoe−/−</td>
<td>↑50%</td>
<td>APLN (apelin) is a peptide secreted by EC as well as other cells including adipocytes. APLN receptors (APLNR) are found on EC, VSMC, and other cells. APLN infusion promotes vasodilation in intact arteries but constriction in endothelium-denuded vessels. In this study, APLN inhibited the vasoconstriction and blocked the enhancement in atherosclerosis and aneurysm formation caused by ANG II infusion. Evidence was presented for a direct interaction between the AT1R and APLNR (326). APLN KO also significantly worsened hypoxia-induced pulmonary hypertension with a reduction in eNOS and KLF2 expression (274).</td>
</tr>
<tr>
<td>APLNR</td>
<td>APLNR−/− apoe−/−</td>
<td>↓34%</td>
<td>APLNR is also known as APJ and AGTRL1 (angiotensin receptor-like 1). Despite results above for apelin, KO of its receptor decreased atherosclerosis with greatly reduced numbers of VSMC in lesions while treatment of cultured VSMC with apelin promoted NOX activity and VSMC proliferation (729). Apelin and APLNR expression were upregulated by carotid ligation and promoted VSMC proliferation and increased neointimal formation (931).</td>
</tr>
<tr>
<td>HSD11B2</td>
<td>HSD11B2−/− apoe−/−</td>
<td>↑1719%</td>
<td>HSD11B2 (11β-hydroxysteroid dehydrogenase 2) inactivates cortisol by converting it to cortisone. Cortisol binds and activates the MR (mineralocorticoid receptor) with affinity similar to aldosterone. Mice were fed Chow diet so atherosclerosis was minimal in apo E null mice but substantial in double KO. Endothelium showed activation with increased VCAM-1 expression. Atherosclerosis was blocked by eplerenone but not amiloride (416).</td>
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2. Activation of PKB/Akt by INSR signaling: a focal membrane event

Upon INSR tyrosine self-phosphorylation, adapters are recruited to phosphotyrosines on the receptor’s cytoplasmic tail. Once bound, such adaptors may become targets of the receptor’s activated tyrosine kinase domain becoming tyrosine phosphorylated themselves. This is the case with IRS proteins (with isoforms IRS1–4). In contrast, serine phosphorylation of IRS can block downstream signaling, one of the mechanisms of both insulin resistance and feedback inhibition.

After tyrosine phosphorylation, IRS1/2 provides suitable binding sites for a type 1A phosphatidylinositol 3 kinase (PI3K) such as PI3Kα. PI3K generates phosphatidylinositol 3,4,5-trisphosphate (PIP3) from phosphatidylinositol 4,5-bisphosphate (PIP2) in cell membranes. This generates a localized gradient of increased PIP3 in the cell membrane surrounding the activated INSR. PIP3 recruits a number of signaling molecules that possess pleckstrin homology (PH) domains or other PIP3-binding domains. IRS proteins themselves contain PH domains which help anchor them to the cell membrane. Importantly, immediate downstream signaling molecules PDK1 (PI3K-dependent kinase 1, also known as PDK1 for PIP3-dependent protein kinase 1) and PKB/Akt are recruited to the membrane by their PH domains. When bound to PIP3, PDK1 self-activates and can then phosphorylate PKB/Akt. PKB/Akt phosphorylates a second phosphorylation site for a type 1A phosphatidylinositol 3 kinase (PI3K) such as PI3Kα. 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portant downstream target of PKB while control of mTORC2 is less well understood.

The designations PKB or Akt are used interchangeably. The name Akt derives from studies in an “A” strain of mouse, originally identified in the 1920s which had given rise by inbreeding to a “k” strain particularly prone to spontaneous thymoma development. These mice were further inbred at Rockefeller University, giving rise to the AKR mouse. A transforming cell line derived from AKR mice gave rise to the name Akt (1674). Active Akt is a major cellular signaling node with many downstream targets. Knockout (KO) of Akt1 (PKBα), the main endothelial isoform, resulted in clearly increased atherosclerosis while bone marrow transplant (BMT) with Akt1-deficient cells had little effect (see Table 1). The proliferative effects of Akt/PKB are sufficient to clearly promote cancer when a mutation results in constitutive activity. Anti-atherosclerotic effects of Akt1 in endothelial cells likely include barer preservation, partial activation of endothelial nitric oxide synthase (eNOS) by direct phosphorylation, mitogenic effects, and inhibition of apoptosis.

Among the pro-proliferative effects of PKB is the inhibitory phosphorylation of Ser9 on glycogen synthase kinase 3β (GSK-3β). After “priming” by a casein kinase, GSK-3β tonically inhibits glycogen synthase. Inhibition of GSK-3β by PKB thus promotes glycogen synthesis. But GSK-3β does much more than inhibit glycogen synthase. Among its many actions are blocking the cell cycle by inhibition of CCND1 (cyclin D1) and blocking protein synthesis by inhibiting the translation factor eukaryotic initiation factor 2B (eIF2B).

Furthermore, GSK-3β is activated by endoplasmic reticulum (ER) stress which is aggravated in endothelial cells by various atherosclerosis risk factors including hyperlipidemia. Recently, blocking GSK-3β by valproic acid was shown to reduce atherosclerosis (1165), providing evidence for at least one downstream target of INSR to explain its anti-atherogenic effect.

PKB further promotes cell survival and proliferation by phosphorylating FOXO (forkhead box O) transcription factors. This inhibitory phosphorylation causes FOXOs to be excluded from the nucleus. FOXO activity can also be affected by acetylation and ubiquitination. Interestingly, the FOXO transcription factors can influence transcription both by binding traditional DNA response elements as well as by protein-protein interactions with other nuclear proteins, independent of DNA binding (369). With regards to the circulatory system, KO of FOXO1 is embryo-lethal due to vessel malformation while conditional KO in adults causes hemangiomas and angiosarcomas due to unchecked proliferation of angiogenic cells. For FOXO3, KO phenotypes are more subtle, but enhanced vessel growth is seen with hypoxia (1326). Control of excess proliferation is exerted by FOXO1/3 through both apoptosis and cell cycle regulatory genes. In Figure 2 are several examples of genes transcriptionally activated or inhibited by FOXO1 or FOXO3. Active nuclear FOXO3 promotes apoptosis primarily by increasing transcription of BIM (Bcl-2-interacting mediator of cell death) and Noxa (a latent word meaning injury or damage), important components of the apoptosis pathway. Entry into the cell cycle is limited by FOXO1/3 by increasing expression of cell cycle inhibitors p21Cip1 (cyclin-dependent kinase interacting protein, encoded by CDKN1A, cyclin-dependent kinase inhibitor 1A) and p27Kip1 (p27 CDK interacting protein) and by blocking transcription of CCND1 (cyclin D1) (369).

Recent work suggests that FOXO1 and -3 have net atherogenic effects in endothelium (1817) but have a protective effect in myeloid cells in part by limiting their proliferation (1818) (see Table 1). FOXO1/3 can also inhibit inflammatory NF-κB signaling, increase production of protective MnSOD (manganese superoxide dismutase, a mitochondrial SOD also known as SOD2), induce DNA repair genes such as GADD45 (growth arrest and DNA damage-inducible gene 45), and generally support longevity (369, 1326). FOXO3 was also found to help limit lung damage from cigarette smoke, primarily due to direct blocking of NF-κB signaling (811). These observations sound a cautionary note for selecting signaling molecules to block as a means to prevent atherosclerosis and illustrate the divergent effects the same genes can have on endothelial versus leukocyte mediated mechanisms.

3. Other adapters and enzymes link INSR to multiple pathways

Other adapters that bind tyrosine phosphorylated INSR (as well as other RTK) include growth factor receptor-bound protein 2 (Grb2), Grb2-associated binder 1 (Gab1), and Src homology collagen-like (Shc), as well as assorted signaling enzymes such as Src (short for sarcoma) (probably by way of IRS for INSR), SH2 domain-containing tyrosine phosphatase (SHP2), and phospholipase C, γ isoform (PLCγ). Much like IRS proteins, Gab1 binds phosphorysorine on active INSR, is then tyrosine phosphorylated by INSR and can then bind PI3K to further enhance PIP3 production. The Src family kinases are an important class of non-receptor tyrosine kinases that can phosphorylate the cytoplasmic tail of RTK as well as other receptors which lack tyrosine kinase activity, or various adapters (such as Shc). Src family kinases thereby act to initiate downstream signaling or amplify the activity of the RTK, particularly its mitogenic activity (for example, through the Shc, Ras, and the MAPK pathway to be discussed below).

The tyrosine kinase Janus kinase (JAK) is brought to INSR by way of the adapter SH2b adapter protein 1 (SH2B1), while a substrate of JAK, signal transducer and activator of transcription 3 (STAT3) appears to be carried by the adapter receptor for activated C-kinase 1 (RACK1) (1630). Upon phosphorylation by JAK tyrosine kinases, signal
transducer and activator of transcription (STAT) proteins form dimers and transmigrate to the nucleus to function as transcription factors. The JAK-STAT pathway represents one of the shortest and most rapidly acting of receptor-activated pathways. Some receptors (such as many for interleukins) are primarily JAK-STAT transducers. When bound to phosphotyrosines on the INSR cytoplasmic tail, PLCγ can cleave membrane-bound PIP2 to release free inositol 1,4,5-trisphosphate (IP3), leaving diacylglycerol (DAG) in the membrane. IP3 binds to and opens the IP3 receptor embedded in the endoplasmic reticulum (ER) membrane, releasing into the cytoplasm calcium stored in the ER. Free calcium can bind calmodulin (CaM) and many other effectors. Furthermore, calcium and DAG together activate conventional protein kinase C serine/threonine kinases (PKCα, βI, βII, and γ). Novel PKC enzymes (PKCδ, ε, η, and θ) are activated by DAG but not calcium. Atypical PKCs, including PKCζ, -ι, and -λ, can be activated by PIP3 or ceramide. All the PKC enzymes are further activated by PDK1 in PIP3-enriched cell membranes.

4. Multiple G proteins are activated by insulin signaling

Small G proteins, also known as GTP-binding proteins or GTPases, are master signaling molecules, serving as on-off switches. G proteins now number some 150 in five families. Most are membrane bound. They mediate the assembly of other signaling proteins or cascades when they are in their “on” or GTP-bound configuration. This they do by facilitating conformational changes in their downstream binding partners without direct enzymatic modifications. When bound with GDP, small G proteins are “off”; they do not bind to effectors. G proteins are activated by guanosine exchange factors (GEFs), which replace GDP with GTP. They are turned off by GTPase activating proteins (GAPs) which supply a missing component to the GTP hydrolytic site inherent to G proteins. The ~800 human G protein-coupled receptors (GPCRs) are essentially GEFs for Gα, a GTPase accompanied by β and γ subunits when bound to the GPCR. In GPCR signaling, GAPs for Gα are often referred to as regulators of G protein signaling (RGS) proteins.

Insulin signaling activates the prototypical small G protein, Ras (named for a transforming oncogene found in rat sarcoma). The Ras superfamily includes Ras, Rho/Rac, Rab, Rap, Arf, Ral, Ran, Rheb, Rad Rit, and Miro families. After INSR activation and IRS1/2 phosphorylation, specific phosphotyrosine residues are recognized on IRS1/2 by the SH2 domain of the adapter Grb2. In the case of EGFR signaling, Grb2 binds directly to activated EGFR. The adapter Shc (Src homology collagen-like) can bind phosphotyrosines on either INSR or EGFR and, once bound, Shc can also act as an intermediary adapter to bind Grb2. Grb2 brings it (bound to SH3 domains) the RasGEF Sos, which then activates Ras. Sos activity is partially dependent on PIP3. Active Sos mediates exchange of GDP for GTP on Ras. Active, GTP-bound Ras can then bind and promote activation of Raf1. Sos also has Rac1 GEF activity. PI3K enzymes possess a Ras binding domain (RBD) which can help approximate the catalytic subunit of the PI3K to its substrate, PIP3, in the cell membrane. This is yet another example of the interconnectedness of these pathways. Once activated by Ras, active Raf1 promotes downstream MAPK signaling (discussed further below).

Ras is permanently and firmly anchored to membranes by covalently attached lipid anchors (farnesol and palmitate). Rho family G proteins, including RhoA (Ras homologous A), Rac1 (Ras-related C3 botulinum toxin substrate 1), and Cdc42 (cell division cycle 42), have geranylgeranyl anchors but shuttle from cytoplasm to membranes, being held inactive and soluble in the cytoplasm with their anchor shielded by various GDP dissociation inhibitor (GDI) proteins. The Src family tyrosine kinases also depend on prenylation and are normally membrane bound. Statins inhibit synthesis of isoprenoid lipid anchors, and this can decrease signaling, especially from Ras, RhoA, and other small G proteins, explaining at least some pleiotropic effects of statins (1356, 1524, 2105). Pharmaceutical inhibition (with manumycin A) of farnesytransferase, which transfers the farnesyl moiety onto Ras, resulted in a 43% reduction in atherosclerotic lesion size and a marked decrease in VSMC proliferation in ApoE-deficient mice (1695).

G proteins of the Rab family (some 60 members in the human genome) control vesicle transport. In muscle and adipose cells, insulin signaling causes storage vesicles with GLUT4 (glucose transporter 4) inserted in their membranes to be translocated by cellular motors along microtubules and then microfibers to the cell membrane where the GLUT4 storage vesicles dock and fuse with the cell membrane, allowing glucose to then enter the cell. This process is largely orchestrated by several Rab G proteins that are found attached to the intracellular GLUT4 storage vesicles. In unstimulated muscle and adipose cells, relevant Rabs are maintained in a GDP-bound, inactive state by the Rab-GAP AS160 (Akt substrate 160 kDa, also known as TBC1D4). After insulin binding, activated Akt/PKB phosphorylates AS160 (Akt substrate 160 kDa), causing it to bind to a 14-3-3 binding protein, thereby rendering it inactive. The Rab G proteins become activated by GTP binding and then direct translocation of the vesicles and membrane fusion, thereby promoting glucose uptake (1009, 1687). While the GLUT4 pathway is lacking in endothelial cells, it illustrates an important signaling tactic, inhibiting an inhibitor (AS160) of G protein signaling.

Another example of inhibiting the inhibitor is the phosphorylation of the TSC1/2 complex (tuberous sclerosis 1 and 2). TSC1/2 is a GAP for the G protein Rheb. Active
Rheb is required for activation of mTORC1 (mammalian target of rapamycin, complex 1) (FIGURE 2). Active PKB phosphorylates and inhibits TSC1/2 leading to GTP binding of Rheb and mTORC1 activation. Active mTORC1 then acts to increase protein synthesis by promoting phosphorylation of the ribosomal protein S6 (by S6K1), activating eIF4E, and inhibiting autophagy. mTOR is opposed in all these actions by AMPK (AMP-activated protein kinase), which senses low cellular energy levels as reflected by increasing AMP. AMPK activates a host of pathways to generally mobilize and increase cellular fuels and energy production while decreasing energy expenditure. AMPK signaling promotes production of new mitochondria and has also been associated with longevity. Interestingly, knockout of at least one isoform of AMPK increased atherosclerosis (see TABLE 1), despite its opposition to at least some aspects of insulin signaling.

In INSR signaling, yet another GEF, whose identity remains somewhat uncertain (312), promotes GTP binding to Rac1, with subsequent activation of NADPH oxidase 1 or 2 (NOX1/2) which then generates superoxide. Rac1 also regulates the cytoskeleton which may further promote proper assembly of NOX components as well as direct GLUT4 vesicle transport. Although superoxide is a potent reactive oxygen species (ROS), its controlled production by NOX enzymes should not be viewed as an inevitably adverse event, but a necessary and normal component of the signaling machinery.

5. Signaling cassettes activated by RTK are mutually supportive: role of oxidases

Note that many of the seemingly disparate pathways activated by INSR are mutually supportive (see FIGURE 2). RTK (and many other receptors) generally activate NOX isoforms and/or calcium-sensitive dual oxidase (DUOX) enzymes (or both) to generate superoxide and hydrogen peroxide, respectively. These ROS reversibly inhibit assorted protein tyrosine phosphatases (PTP) as well as the lipid phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome ten). PTEN acts to cleave the 3 phosphate on PIP3. PTEN and the various tyrosine phosphatases can be so active that, without inactivation, no signal can be propagated. Therefore, most cellular activating systems (whether proinflammatory or anti-inflammatory and including various growth factors, cytokines, antigen recognition, ANG II, thrombin, and even insulin) simultaneously stimulate ROS production to prolong their signals. ROS thus have the important signaling task of inhibiting the inhibitors of RTK (and other) signal transmission. This is accomplished by generating far less superoxide than fully activated NOX2 in neutrophils and other phagocytes, although the same NOX2 (and other NOX isoforms) are found on endothelial cell membranes.

The lipid phosphatase PTEN is among the most important signal terminating enzymes. By removing phosphate from the inositol-3 position of PIP3, PTEN acts in direct opposition to PI3K. Because PKB/Akt requires PIP3, PTEN also inhibits cell proliferation and promotes apoptosis, yet it is also anti-inflammatory in various circumstances. Indeed, many tumors express diminished PTEN, allowing excess PIP3 accumulation and PKB activity which promotes cell proliferation and survival. VSMC-specific knockout of PTEN leaves PI3K unopposed. Vascular injury in PTEN KO animals results in markedly increased neointimal formation (from excessively proliferating VSMC) and greatly increased production of cytokines by the activated VSMC (569, 1292). In endothelial cells and macrophages, both proinflammatory (e.g., priming of PKC, increase foam cell formation in macrophages) and anti-inflammatory pathways (e.g., increased eNOS phosphorylation and activation) are, in part, dependent on Akt/PKB activity. In one study, the plant alkaloid berberine was thought to increase atherosclerosis in ApoE null mice by supression of macrophage PTEN causing an increase in the expression of scavenger receptor SR-A, uptake of modified LDL, and foam cell formation (1027). Conversely, upregulation of PTEN in endothelial cells, either by specific alkaloids (810, 1816) or by PPARy agonists (810, 838, 909) substantially attenuated expression of the adhesion molecule VCAM-1 in response to the inflammatory cytokine TNF-α and decreased monocyte adherence while inhibiting endothelial proliferation and angiogenesis. These findings suggest a complex and difficult to predict role for PTEN in relation to atherogenesis. Perhaps not surprisingly, the overall effect on atherosclerosis of whole-body heterozygous PTEN KO was neutral (64).

Note that many of the seemingly disparate pathways activated by INSR are mutually supportive (see FIGURE 2). RTK (and many other receptors) generally activate NOX isoforms and/or calcium-sensitive dual oxidase (DUOX) enzymes (or both) to generate superoxide and hydrogen peroxide, respectively. These ROS reversibly inhibit assorted protein tyrosine phosphatases (PTP) as well as the lipid phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome ten). PTEN acts to cleave the 3 phosphate on PIP3. PTEN and the various tyrosine phosphatases can be so active that, without inactivation, no signal can be propagated. Therefore, most cellular activating systems (whether proinflammatory or anti-inflammatory and including various growth factors, cytokines, antigen recognition, ANG II, thrombin, and even insulin) simultaneously stimulate ROS production to prolong their signals. ROS thus have the important signaling task of inhibiting the inhibitors of RTK (and other) signal transmission. This is accomplished by generating far less superoxide than fully activated NOX2 in neutrophils and other phagocytes, although the same NOX2 (and other NOX isoforms) are found on endothelial cell membranes.

The active NOX2 complex consists of several subunits (119). NOX2 is the catalytic subunit (also known as gp91phox). It is permanently bound to the membrane with six transmembrane domains while its cytoplasmic COOH terminus binds FAD and NADPH. NOX2 is found constitutively bound to the p22phox subunit which stabilizes NOX2. NOX2 activation requires phosphorylation of the regulatory p47phox subunit which then moves from the cytoplasm to bind the p22phox subunit. This p47phox “organizer” subunit brings with it p67phox (the “activator” subunit) and p40phox (a modulatory subunit) to the membrane-bound complex. As noted above, activation of NOX2 also requires the active, GTP-bound form of Rac1 which first binds NOX2 then p67phox. Phosphorylation of p47phox may be accomplished by PKC enzymes activated by DAG supplied by PLCγ, also activated by INSR. As noted above, INSR can supply active Rac1. In endothelial EGRF signaling, Rac1 can be activated by Vav2 (Vav1 is found primarily in hematopoietic cells). The GEF activity of Vav2 is partially dependent on PIP3, again supplied by the PI3K cassette. There are many other Rac1 GEFs that are not dependent on PIP3. PIP3 also mediates binding of the p40phox subunit of NOX to the cell membrane.
While a low level of NOX activity is expected to be important for the apparently anti-atherosclerotic activity of endothelial INSR signaling, higher NOX activity is frequently associated with atherosclerotic plaque. Such pro-atherogenic NOX signaling is found in activated macrophages and other leukocytes as well as activated endothelial cells. Thus genetic deletion or impairment of NOX2, p47phox, and the main source of NADPH utilized by NOX enzymes, glucose 6 phosphate dehydrogenase (G6PDH), were all found to clearly reduce atherosclerosis (see **TABLE 1**). In bone marrow transplant studies in ApoE-deficient mice, deficiency of p47phox in the artery wall with normal bone marrow resulted in a 66% reduction in atherosclerosis and marked reduction of VCAM-1, ICAM-1, and P-selectin adhesion molecules expressed on both endothelial cells and VSMC as well as relative insensitivity to thrombin signaling. Transplant of p47phox-defective, ApoE-deficient marrow into ApoE-deficient mice with wild-type p47phox resulted in 53% attenuation of atherosclerosis, suggesting both vascular wall and macrophage production of superoxide contributes importantly to atherosclerosis progression (1872).

NOX2 is upregulated in human coronary arteries from patients with CAD, even in areas without lesions (682). NOX2 deficiency causes X-linked granulomatous disease. Normal human subjects all experienced impairment of flow-mediated dilation after 20 min of arm ischemia while patients with NOX2 deficiency (or p47phox deficiency) did not (1079), again demonstrating relevance of NOX2 in short-term endothelial function. Endothelial NOX5 expression was sevenfold higher in early human coronary atherosclerotic lesions and contributes to ROS production (680). The NOX5 gene was apparently lost in rodents and hence cannot be studied by KO in mice. Hyperlipidemia appears to contribute to age-related increases in arterial wall ROS production, since endothelial NOX2 expression and superoxide levels markedly increased with age in ApoE-deficient mice but not in wild-type mice.

NOX1 and NOX4 are also present in endothelial cells and contribute to ROS production (1911). NOX4 is constitutively active and seems little affected by at least some inflammatory signals (although possibly induced by others) while NOX1 plays a greater role in VSMC superoxide production. In some circumstances, NOX4 may actually be anti-inflammatory through chronic, background superoxide production leading to stimulation of cellular antioxidant defenses (1566). Remarkably, p47phox KO reduced endothelium-dependent vasodilation in response to VEGF due to impaired PI3K-Akt-eNOS signaling (515), suggesting a need for balance in ROS-producing pathways.

**6. Signaling cassettes activated by RTK are mutually supportive: role of Src and other components**

Other mutual dependencies of signaling cassettes are apparent in **FIGURE 2**. The activity of PI3K is at least partially dependent on active Ras. The tyrosine kinase Src (or another Src family kinase) is activated by INSR (and EGFR) through the intermediary action of SHP2. Src amplifies EGFR activation by phosphorylating EGFR tyrosines 845 and 1101, Gab1, STAT3, and STAT5a/b. Src also phosphorylates ADAM17, further activating its “sheddase” activity, which thereby releases more HB-EGF (89, 1004). Similar amplification likely occurs with INSR. Perhaps not surprisingly, insulin signaling through INSR acts synergistically with EGFR to increase the mitogenic effects of EGFR signaling (180). Not shown in **FIGURE 2** are a number of INSR-interacting proteins (such as Cbl: Casitas B-cell lymphoma) or SOCS (suppressor of cytokine signaling) that initiate feedback inhibition and downregulation (89). Nor are the complex pathways shown that regulate transport of GLUT4-containing vesicles to and their fusion with the cell membrane, since GLUT4 is not expressed on endothelial cells.

**B. An Introduction to MAPK Signaling**

1. Why MAPK signaling cassettes?

As noted above and in **FIGURE 2**, MAPK signaling is one of the major pathways activated by RTK ligation. **FIGURE 3** illustrates some of the essential features of MAPK signaling in this context. MAPK modules can be thought of as signaling bottlenecks that collect assorted signals from receptors, G protein switches, ROS, integrins, and other cellular sensors and then transduce these diverse signals into a highly controlled off/on signal which is passed on to numerous cellular effectors and feedback controls (1141). For example, ERK1/2, the prototypical terminal MAPK, may phosphorylate over 100 proteins. These downstream effectors mediate critical cellular functions including metabolism, gene transcription in response to challenges, cell architecture, differentiation, cell survival, proliferation, and apoptosis. MAPK modules may be seen as key, controlling guardians supervising these critical pathways. While only a few MAPK-related gene knockouts have been directly tested for effects on atherosclerosis, there are numerous interventions which affect cell function through MAPK pathways. Furthermore, MAPK are majorly involved in angiogenesis (607).

Each MAPK module consists of a series of three consecutively activated protein kinases, typically held in juxtaposition by a scaffold protein. Other regulatory proteins, including deactivating phosphatases, are also part of the complex. MAPK designates the terminal serine/threonine kinase. The terminal MAPK is activated by a MAPKK (MAPK kinase or MAP2K, also called a MEK for MAPK/ERK kinase). This intermediate kinase may itself function as a scaffold and is phosphorylated by an upstream MAPKK (MAPKK kinase, or MAP3K also known as a MEKK or MEK kinase). Terminal MAPK activation requires two...
phosphorylations in two separate hits on a Thr-X-Tyr (TXY) motif in the activation loop. Thus MAP2K are rather exceptional dual-function kinases with both serine/threonine and tyrosine kinase activities. Similarly, MAP2K activation requires the phosphorylation of two closely spaced serines (SXXXS motif). MAP3K activation is far more diverse and generally requires multiple activating signals with modulation by inhibitory modifications.
Mammalian terminal kinases include ERK(1–7) (with ERK1 and ERK2 nearly indistinguishable in function), JNK(1–3), and p38(α, β, γ, and δ). Traditionally, ERK1/2 are activated by mitogens while p38 and JNK are activated by cell stress or cytokine-generated signals. JNK signaling, in particular, can lead to apoptosis. In endothelial cells, ERK5 signaling is generally anti-inflammatory and cytoprotective (see sect. IVD). Kinases that can phosphorylate MAP3K are sometimes referred to as MAP4K and may be involved in multiple interacting pathways.

The unique architecture of MAPK modules, with their scaffolds, associated phosphatases, and requirements for two closely spaced phosphorylations to activate the terminal kinases all work together to impart uniquely useful kinetic properties, indicated in Figure 3. Thus MAPK modules remain inactive, acting as noise filters at low level inputs, become rapidly activated once inputs rise above a specific level, and then display a stable, limited output activity even when inputs rise to very high levels (1140). Furthermore, the scaffold proteins (and other regulatory proteins) can dampen or amplify the output depending on cell status or context (1067, 1742). All these properties are highly desirable features of a biochemical switch controlling crucial downstream processes and help explain the preservation of MAPK pathways in essentially all eukaryotic organisms. Furthermore, cellular effects vary markedly depending on cell type, duration, and location of MAPK signaling. For example, transient cytosolic activation of ERK can lead to cell proliferation, while sustained nuclear activity can cause terminal differentiation (933).

3. Activation of Raf1, a prototypical MAP3K, and other activators of MAPK pathways

Binding of active, GTP-bound Ras to Raf1 is necessary but not sufficient for Raf1 activation. Indeed, the structure of Raf1, shown in Figure 3, illustrates the multiple potential inputs to a MAP3K in general. Activating phosphorylations of specific serine (S), threonine (T), or tyrosine (Y) residues, together with potential (not necessarily exclusive) activating protein kinases are shown in the black arrow boxes. The red arrow boxes show inhibiting phosphorylations. Additional phosphorylations, which are not shown in Figure 3, can add still greater complexity and feedback inhibition (1510). Phosphorylations at S43, S233, and S259 sterically inhibit Raf-1 binding to GTP-Ras and promote, instead, binding to the adapter/chaperone protein 14-3-3 (named for the separation fraction where it was discovered). Various 14-3-3 isoforms often hold proinflammatory client proteins like Raf1 inactive in the cytoplasm as shown in Figure 3 (1537, 2103, 2125). There is a suggestion that upregulation of 14-3-3 proteins may be protective in atherosclerosis, although assignment of specific roles for the different isoforms is difficult (218, 1052). Remarkably, however, phosphorylation of S621 on Raf1 creates a binding site for 14-3-3 which is mandatory for Raf1 activity (by providing a binding site for ATP and a shield against S621 dephosphorylation) (419). Once active, Raf1 phosphorylates MEK1 or MEK2 which can then phosphorylate and activate the terminal MAPK, ERK1, or ERK2.

In Figure 3 only one MAP3K is shown for the JNK and p38 modules, whereas, in reality, a number of MAP3K can activate either or both JNK or p38. These MAP3K include MEKK1–4, MLK2/3 (mixed lineage kinases 2 and 3, also known as MAP3K10 and MAP3K11), ASK1 (apoptosis signal-regulating kinase 1), NIK (NF-κB-inducing kinase or MAP3K14), and TAK1 (TGF-β-activated kinase-1 or MAP3K7). A number of these MAP3K as well as their activating MAP4K are involved with NF-κB activation as well, illustrating important network behavior or cross-talk between MAPK and NF-κB activation pathways. Surprisingly, KO of ASK1, either whole body or in bone-marrow derived cells only, resulted in an increase in atherosclerosis with greater macrophage accumulation and less macrophage necrosis (see Table 1).

Several MAP4K are particularly relevant to endothelial cell activation. These include PKCζ (which has multiple targets), (GCK; or MAP4K2), and PAK1/2 (p21 activated kinase 1 and 2). Flow-induced PAK activation in endothelial cells apparently activates JNK through the MAP2K MKK4 (283, 692, 1353). JNK induces bone morphogenic protein 2 (BMP2), BMP4, and Toll-like receptor 4 (TLR4) and has numerous other proinflammatory and pro-apoptotic effects in endothelial cells (285) yet only JNK2 appeared to be atherogenic (see Table 1).

4. MAPK, MAPK targets, and atherosclerosis

Once active, one of the targets of ERK1/2 (and p38) is MSK1 (mitogen- and stress-activated protein kinase 1). MSK1 phosphorylates serine 276 of the NF-κB p65 subunit after translocation to the nucleus (1467). This is a key modification of p65 which facilitates CBP/p300 binding and...
acetylation of p65 Lys310, allowing full transcriptional competency for NF-kB (see below); yet another example of cross-talk between MAPK and NF-kB signaling. Also indicated in Figure 3 are the generally proinflammatory activities of JNK and p38. Thus JNK activates transcription factors including early growth factor 1 (Egr1), activating transcription factor 2 (ATF2), and activator protein-1 (AP-1), which contains either c-Fos, c-Jun, or both. Egr1 and AP-1 generally induce proinflammatory gene transcription. ApoE-deficient mice with Egr1 knockout had a marked reduction in atherosclerosis (see Table 1).

Activation of MAPK p38 in areas of disturbed flow also appears to be an important means of ATF2 and AP-1 activation (529, 2011), thus priming endothelial cells for subsequent activation. Another target of p38 and ERK1/2 phosphorylation is MAPK-activated kinase 2 (MK2) which can phosphorylate the p47phox to facilitate NOX activation and ROS production. MK2 also appears to be involved in mRNA stabilization for a number of proinflammatory proteins whose mRNA intrinsically has rapid turnover due to AU-rich elements in their 3′ untranslated regions (973). MK2 KO reduces atherosclerosis (Table 1) but, unfortunately, also increases susceptibility to infection. Paradoxically, however, p38 KO nonsignificantly increased atherosclerosis (see below).

MAPK phosphatase 1 (MKP-1) was found to be expressed exclusively in arterial regions protected by high laminar flow in mouse aorta (2056), but the mechanism of MKP-1 induction by flow is currently unknown. MKP-1 is a dual-specificity phosphatase that can deactivate all MAPK but preferentially p38 and JNK. While inhibition of these MAPK would seemingly be anti-inflammatory and therefore atheroprotective, MKP-1 deficiency was, in fact, found to decrease atherosclerosis (820, 1612). MKP-1 KO mice transplanted with wild-type bone marrow had slightly greater (though nonsignificant) atherosclerosis compared to wild-type mice receiving wild-type bone marrow, suggesting only mild, if any, effects due to MKP-1 deficiency in the vessel wall (1612). However, macrophages from MKP-1-deficient mice were found to have impaired ERK1/2 expression and marked defects in their ability to spread and migrate, and reduced MCP-1 production, resulting in a reduction in lesion macrophage content (820, 1612).

In several studies of MAPK signaling components, the direction of the effect seemed paradoxical. Thus ASK1 KO clearly increased atherosclerosis, with a lesser (nonsignificant) effect from macrophage-specific p38 KO, while MKP-1 KO decreased atherosclerosis. These unexpected results seemed to occur when the endothelial effect would have been predicted to be anti-inflammatory and anti-atherogenic (or proinflammatory and proatherogenic), while at the same time, a seemingly opposite effect was seen in macrophages or other leukocytes. MAPK generally have strong prosurvival effects on macrophages and can have varied effects on scavenger receptor expression and foam cell formation. The resulting effects on atherosclerosis can be nuanced and difficult to predict.

In MKP-1 deficiency, an unknown mechanism led to impaired ERK1/2 expression which was linked to defective migration, an unexpected anti-inflammatory effect on macrophages (820, 1612). In ASK1 KO, there was less early macrophage apoptosis and hence larger lesions (2005). There was also less macrophage necrosis which might actually be an advantage in more advanced lesions (although this possibility was not tested). If ASK1-deficient endothelial cells were less prone to inflammation, this tendency was apparently less important for lesion size than the macrophage effects. This may be seen as a disadvantage to whole-body KO studies in general, since differences in endothelial and macrophage effects, which may act in the opposite direction, may not be separable.

Endothelial insulin signaling has been proposed to act as a two-edged sword, with the potentially proinflammatory Grb2-Sos-Ras-Raf1-MAPK cascade and production of the vasoconstrictor endothelin-1 (ET-1) on the one hand and the relatively protective, antiapoptotic, anti-inflammatory, and vasodilatory PKB-eNOS pathway on the other (1251, 1432). The finding of increased atherosclerosis with IRS2 knockout in ApoE-deficient mice which was directly proportional to plasma insulin concentrations is supportive of this view (637). Based on the increased atherosclerosis seen in mice with endothelial-specific INSR deletion, the overall endothelial effect of normal insulin signaling can be taken as protective. However, as with the unexpected findings for MAPK signaling noted in the prior paragraph, macrophage-specific deletion of INSR decreased atherosclerosis (Table 1). In both cases, the direction of the effect could potentially be explained by survival effects for the particular cell type. Moreover, the insights gained would probably have been obscured if only whole body KO experiments had been conducted.

C. NF-kB Activation

1. General features of NF-kB signaling

Some departure from the “outside-in” order of presentation is taken here to first introduce NF-kB itself and to illustrate the central significance of this master proinflammatory, antiapoptotic transcription factor. Signaling pathways related to NF-kB are exceedingly complex. Indeed, in the year 2012 alone, there were 5,683 publications listed in PubMed that included “NF-kB” in an indexed field. Several excellent, recent reviews are available (1639, 1841, 1944). Like most transcription factors, NF-kB is a dimer. Most commonly, RelA (also known as p65) is paired with p50.
Other Rel subunits (RelB, and c-Rel) and the p52 subunit can also form NF-κB transcription factors. The subunit p50 is the product of the NF-κB1 gene and has p105 as its precursor (processed to p50 constitutively) while the NF-κB2 gene encodes p52 and its precursor, p100. In nonstimulated cells, NF-κB dimers are held inactive in the cytoplasm by one of several IκB (inhibitor of κB), most frequently IκBα and IκBβ while other classes of IκB can serve different functions (2009). In addition, the p100 precursor of p52 binds RelB and holds it inactive. The p100 precursor also binds and inhibits other NF-κB dimers by way of the same domain shared by IκB proteins (ankyrin repeats). In the canonical pathway, IKK2 (IκB kinase 2 or IKKβ) is activated and targets IκB for phosphorylation, ubiquitination, and subsequent proteosomal degradation, releasing NF-κB dimers for transport to the nucleus. IKK1 (IKKα) is generally controlled by the noncanonical pathway (see below) and leads to processing of p100.

In endothelial cells, active NF-κB induces transcription of cell adhesion molecules such as ICAM-1, VCAM-1, and E-selectin, as well as several chemokines and cytokines including MCP-1, M-CSF, GM-CSF, IL-1β, IL-6, IL-8, and TNF-α. Given the generally pro-inflammatory gene targets of NF-κB, it is not surprising that many elements of upstream signaling leading to NF-κB activation have been found to be atherogenic (see TABLE 1). Nevertheless, NF-κB also has a number of antiapoptotic targets, including IκBα (representing a form of feedback inhibition), cFLIP (cellular FLICE inhibitory protein), cIAP1/2 (cellular inhibitor of apoptosis 1 and 2), XIAP (X-linked inhibitor of apoptosis), the adapter/binding protein 14-3-3 (some isoforms), A20, Cezanne (cellular zinc-finger anti-NF-κB enzyme), COX-2, and eNOS. Why might NF-κB be both proinflammatory and anti-apoptotic? Teleologically, one might reason that the body’s soldiers and sentinels (leukocytes and endothelial cells) should protect themselves while fighting the enemy.

The NF-κB signalosome is somewhat analogous to the MAPK module, especially since serine/threonine kinases that activate the IKK complex, including TAK1, NIK (NF-κB inducing kinase), and MEK3, can all serve as MAP3K. IKK1 and IKK2 are comparable to MAP2K and even share the same SXXXS activating motif. Finally, targeting of IκB proteins by IKK1/2 leads to transcription factor activation. The pathways even share several common MAP4K (such PKCζ) (87, 796).

A focal point of NF-κB signaling is activation of IKK2 and IKK1. Not only does active IKK2 phosphorylate IκB, targeting it for ubiquitination and degradation, but IKK2 has a number of other targets as well, both cytosolic and nuclear (1325). Importantly, active IKK2 serine phosphorylates insulin receptor substrate 1 (IRS-1) and thereby promotes insulin resistance in various tissues. Promiscuous activation of IKK2 and NF-κB in adipose, macrophages, pancreas, and liver may be one of the major features of metabolic syndrome and type II diabetes and may partly mediate some of the associated cardiovascular risks (79, 431, 631). Liver-specific overexpression of human IKK2 in ApoE-Leiden hyperlipidemic mice led to a modest increase in IKK2 activation in liver and only a mild increase in systemic susceptibility to inflammatory signaling. Nevertheless, there was a greater than twofold increase in atherosclerosis (1975). Perhaps some of the increased atherosclerosis was promoted by a transient and mild increase in plasma very-low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) levels, but most of the effect was thought to be due to increased NF-κB activation. Inhibition of IKK2 (but not IKK1) can be achieved by pharmacological levels of salicylic acid. The nonacetylated forms of salicylate do not inhibit COX enzymes and appear to primarily exert their anti-inflammatory and insulin-sensitizing effects as well as improvement of endothelial function through IKK2 inhibition (1417, 2040, 2051). Salicylate treatment decreased atherosclerosis in LDLR−/− mice by ~40% (817). Furthermore, salsalate (a dimer of salicylic acid) administration decreased insulin resistance and inflammatory markers in obese young adults (530) and was recently shown to improve glycemic control and increase adiponectin levels in adult type II diabetics (630).

2. NF-κB translocation: general features

Conversion of the inactive, bound, cytosolic NF-κB to a fully functional transcription factor involves two fundamental steps, translocation to the nucleus, and transactivation. For translocation, NF-κB must be released from its inhibitors and move to the nucleus. This occurs by canonical, noncanonical (alternate), and atypical pathways. In FIGURE 4, canonical and noncanonical activation is illustrated downstream of TNF receptor 1 (TNFR1) and TNFR2, respectively. Both the canonical and noncanonical pathways converge on the IKK complex, composed of the kinases IKK1 (also referred to as IKKα) and IKK2 (or IKKβ) bound to their scaffold NEMO (NF-κB essential modulator, also known as IKKγ). The canonical pathway leads to phosphorylation and activation of the IKK2 subunit, generally by the serine/threonine kinase TAK1. When activated, IKK2 phosphorylates IκB proteins (at serines 32 and 36 for IκBα), marking them for ubiquitination with lysine 48-linked (K48) chains. K48 ubiquitination targets IκB for degradation by the 26S proteosome, thereby releasing the NF-κB dimer for transport to the nucleus. The noncanonical pathway is characterized by activation of NIK and IKK1 activation.

3. NF-κB translocation by the canonical pathway: TNFR1 signaling

Upon ligation of TNFR1 by soluble or membrane-bound TNF-α, the adapter TRADD (tumor necrosis factor recep-
Activated NOX2 produces superoxide within the endosome which can escape through anion channels such as chloride channel 3 (CLC3) into the cytoplasm where it is rapidly converted to hydrogen peroxide by superoxide dismutase 1 (SOD1). Interestingly, oxidation of extracellular cysteines on TNFR1 and TNFR2 (which are intraendosomal after endocytosis) seems to enhance binding and subsequent signaling by TNFα (1363). ROS and especially peroxynitrite increase PKCζ activity as well (551).

Receptor-bound TRADD binds TRAF2 which recruits cIAP1/2 (cellular inhibitor of apoptosis protein 1 and 2) and RIP1 (receptor-interacting protein 1) to the cytoplasmic tail of TNFR1, forming “complex I.” ROS derived from NOX2 activated in the prior step enhances binding of TRAF2 to TRADD, probably by alterations of cysteine residues (1321). This model is consistent with hydrogen peroxide as an enhancer, but not necessarily an initiator of NF-κB signaling (1345). It should be noted that creation of a microdomain with high hydrogen peroxide/ROS concentrations immediately surrounding the receptor complex may provide adequate ROS stimulation without an absolute need for endosome formation. Some experimental evidence suggests that endosome formation may not be required for activation.

Subsequent downstream canonical signaling depends on E3 ubiquitin ligases. Indeed, ubiquitination is of key importance in all TNF receptor superfamily signaling. Most readers will be familiar with ubiquitin tagging of proteins to target them for proteosomal degradation. Such tagging usually involves assembly of so-called “K48” (lysine 48) ubiquitin chains on the targeted protein. More recently, the formation of alternative ubiquitin structures serving very different functions has been recognized. Three classes of ubiquitinases are required for ubiquitination (120, 1944). E1 ubiquitin activating enzymes utilize ATP to attach the glycine COOH terminus of ubiquitin to an E2 enzyme by a high-energy thioester bond. Ubiquitin-charged E2 enzymes (ubiquitin conjugases) attach the COOH terminus of ubiquitin to the target protein and extend the ubiquitin chain. E3 enzymes (E3 ubiquitin ligases) join in a complex with E2 enzymes, directing the E2 enzyme to a particular protein target. E1 and E2 enzymes are not shown in Figure 2. There are only a few E1 enzymes, less than 40 E2 conjugases, but over 600 E3 ubiquitin ligases in the human genome, providing greater signaling diversity than even the 518 or so known protein kinases (1036). TRAF proteins as well as cIAP1/2 are E3 ubiquitin ligases. When newly added ubiquitins are linked repeatedly to ubiquitin K48 at the tail of the growing chain, the protein is targeted for degradation. In contrast, if newly added ubiquitin is linked repeatedly to lysine 63 (K63) of the terminal ubiquitin, a dynamic scaffold is produced which serves to assemble downstream signaling components. Similarly, linkage to the NH2 terminus of the growing ubiquitin chain by LUBAC (linear ubiquitin chain assembly complex) yields so-called linear ubiquitin chains which also provide a signaling scaffold (143).

After assembly of complex 1 (TRADD, TRAF2, cIAP1/2, RIP1, and associated E1 and E2 enzymes), TRAF2 and cIAP1/2 decorate RIP1 and themselves with K63 ubiquitin chains. These K63 chains, as well as linear ubiquitin chains synthesized by LUBAC, emerge from TRAF2, RIP1, and cIAP1/2 like a growing mop from the activated receptor complex. K63 ubiquitin chains provide a transient and dynamic scaffold for strategic spacial juxtaposition of downstream adapters, kinases, ubiquitinases, and signaling targets, ultimately mediating the activation of the IKK complex and release of NF-κB. Thus adapters TAK1-binding protein 2 (TAB2) and NEMO bind the growing K63 chains bringing the IKK complex (NEMO, IKK1, and IKK2) into

**FIGURE 4.** Introduction to NF-κB signaling. Tumor necrosis factor-α (TNF-α)-mediated activation of NF-κB signaling is representative of the TNF receptor superfamily. Although not shown, MEKK1 recruitment and activation may be dependent on K63 ubiquitinated TRAF2 and RIP1. Also not shown is the E3 ubiquitin ligase, SCF-βTrCP (S-phase kinase-associated protein 1, Culin1, F-box protein complex-β-transducin repeat-containing protein) which mediates K48 ubiquitin chain attachment onto κBα after κBα is phosphorylated by IKK2. Sphingosine-1-phosphate (S1P) is a required cofactor for TRAF2 to function as a K63 E3 ubiquitin ligase. See text for additional details and abbreviations.
juxtaposition with active TAK1. Additional ubiquitination of TAK1 and NEMO seems to increase TAK1 activity and amplify the signal (498). Phosphorylation of IKK2 by TAK1 activates IKK2 which phosphorylates IκBα, marking it for K48 ubiquitination and proteosomal degradation. The decrease in IκBα liberates RelA:p50 dimers to translocate to the nucleus. At the same time, removal of IκBα exposes Rel A Ser276 which becomes a target of activated PKA subunits (designated PKAc). Translocation of RelA:p50 to the nucleus ensues, apparently mediated by the adapter protein A-kinase-interacting protein 1 (AKIP1) with enhancement by hydrogen peroxide (588).

Different complexes held together by ubiquitin K63 chains can separate from the receptor and continue signaling in the cytoplasm (as can complex I described above). Deubiquitinating or ubiquitin editing enzymes such as A20, Cezanne, and cylindromatosis (CYLD) control inflammation by degrading K63 chains and creating in their place new K48 chains, thereby targeting key signaling components for proteosomal degradation (1875). Not surprisingly, A20 deficiency increases atherosclerosis (see TABLE 1).

IκBα virtually disappears from the cytoplasm within 10–15 min of TNFR1 activation (498). Maximal nuclear translocation of NF-κB and DNA binding is seen by 15–60 min (1740), and some induced gene transcription products (including new synthesized IκBα reappearing in cytosol) become detectable after ~1–2 h (1741). ICAM-1 appears in 2–4 h (2109), and a number of other targets such as cIAP1/2 and X-linked inhibitor of apoptosis (XIAP) increase for at least 24 h (1740).

Not shown in FIGURE 4 following TNFR1 activation is the more delayed or alternative formation of complex II which contains TRADD, RIP1, Fas-associated death domain (FADD), and pro-caspase 8. Activation of caspase 8 leads to caspase 3 activation and apoptosis. Complex II activation may require receptor endocytosis through clathrin-coated pits (as opposed to the caveolae with caveolin shown in FIGURE 4). Endocytosis is followed by NOX-mediated generation of superoxide which is needed for activation of lysosomal acid sphingomyelinase and ceramide synthesis on the outer leaf of the plasma membrane or the intraendosomal side (370, 1424, 1765). Prolonged exposure to high levels of ROS and excessive NO from iNOS activation seem to favor complex II activation and apoptosis. The ubiquitination of RIP1 seems to be a key determinant of complex II formation. The adaptor ABIN1 (A20 binding and inhibitor of NF-κB) binds ubiquitin chains on RIP1 and inhibits caspase 8 binding to FADD, thus preventing formation of complex II (1354). In contrast, free or nonubiquitinated RIP1 promotes formation of complex II and apoptosis. Increased expression of ubiquitinases such as USP2a that target RIP1 K63 ubiquitin chains seems to enhance apoptosis following TNF1 engagement (1113).

4. The noncanonical pathway for NF-κB translocation

The noncanonical or alternative pathway is said to respond more slowly to upstream signals and requires continuing protein synthesis; however, activation is more persistent. Noncanonical NF-κB translocation is dependent on NIK activation. Fewer receptors (including TNFR2, CD40, BAFF, and lymphotoxin β receptors, among others) are thought to activate the alternative pathway compared with the canonical pathway but age-related accumulation of tissue-bound TNFα, the only ligand for TNFR2 (1459), may cause prolonged activation.

TNFR2 is highly expressed on vascular endothelial cells (1958). When ligated, TNFR2 probably also forms a complex with NOX in endosomes like TNFR1. In unstimulated cells, NIK is scarce due to a constitutive K48 ubiquitination and proteosomal degradation. The ongoing K48 ubiquitination is carried out by a cytoplasmic complex of NIK, TRAF3, TRAF2, and cIAP1/2 in which NIK is targeted by cIAP1/2. TNFR2 ligation causes TRAF3 to bind the TNFR2 cytoplasmic tail, disrupting the complex with NIK and redirecting cIAP1/2 E3 ligase activity to TRAF2 and TRAF3. Their subsequent depletion leads to increasing accumulation of active NIK (1459). Reduced TRAF2 and TRAF3 also appears to promote complex II formation if TNFR1 is ligated (1824). Thus TNFR2 signaling may promote apoptosis through TNFR1. Full NIK activation may require phosphorylation and K63 ubiquitination (141, 853). Active NIK phosphorylates IκK1 which, in turn, phosphorylates the p100 precursor of p52. K48 ubiquitination of p100 serves as a marker for only partial proteosomal degradation, the product being mature p52. Reduction of p100 and simultaneous provision of p52 yields functional RelB:p52 dimers which translocate to the nucleus. Depletion of p100 also releases RelA:p50 dimers (2058). TNFR2 ligation additionally supports angiogenesis probably by release of constitutively bound bone marrow tyrosine kinase on chromosome X (BMX), a tyrosine kinase which transactivates VEGFR2 and may lead to activation of PI3K (1090).

High NIK activity not only activates IκK1 but also IκK2, apparently by IκK1 phosphorylation of IκK2 (686). When activated, IκK1 as well as IκK2 have effects well beyond NF-κB translocation with nuclear phosphorylation targets including the RelA subunit as well as other transcription factors and cytoplasmic signaling proteins (355, 623, 1405).

The contribution of NIK to NF-κB activation is often underappreciated. Both TNFR1 and TNFR2 seem to mediate adhesion molecule expression, yet TNFR2 appears to have a greater role for E-selectin and ICAM-1 (275). Deficiency of either TNFR1 or TNFR2 strongly inhibited atherosclerosis in hyperlipidemic mice (276) (see TABLE 1). Prolonged cytokine or other signaling leading to ROS generation (as...
from ANG II, IL-1, or exposure to reactive aldehydes) seems to lead to greater NIK activation (1030, 1069, 1440). Depletion of cIAP1/2 predictably leads to an increase in NIK. Such depletion occurs when apoptotic signals release second mitochondria-derived activator of caspase (SMAC) which causes cIAP1/2 auto-ubiquitination and rapid degradation (1639). With the use of an informatics approach, NIK signaling was predicted to be a major activator of NF-κB in the artery wall 3 or more hours after initial TNFα stimulation (2024). Additional details of other TNF family receptor signaling, including IL-1R, TLR4 (similar to TLR2 stimulation (2024). Additional details of other TNF family receptor signaling, including IL-1R, TLR4 (similar to TLR2

Only a few TRAF proteins have been targeted for knockout to study effects on atherosclerosis. Surprisingly, TRAF1 KO reduced atherosclerosis in LDLR null mice even though TRAF1 had been thought to inhibit TRAF2, particularly in TNFR2 signaling (1217). The apparently pro-atherogenic effect of TRAF1 may possibly be explained by observations on oligomer formation. TRAF2 functions as a homotrimer in its binding and activation of cIAP1/2 to form K63 chains on RIP1. TRAF1 can replace a single TRAF2 chain resulting in a heterotrimer that binds cIAP1/2 more avidly than the TRAF2 homotrimer, thereby promoting more active signaling (2097).

The finding of increased atherosclerosis in LDLR-null mice with TRAF5 deficiency was also surprising (1218). As TRAF5 may substitute for TRAF2 in the alternative pathway, TRAF5 deficiency may possibly lead to increased liberation of NIK. CD40, the receptor for CD40L has mixed canonical and alternative pathway capabilities through a single proximal binding site for TRAF6 and 2 distal sites that can bind TRAF1, 2, 3, or 5. Genetic disruption of the TRAF6 binding site nearly abrogated atherosclerosis (90%) in hyperlipidemic mice accompanied by an increase in IL-10 production by macrophages shifted toward anti-inflammatory M2 differentiation. In contrast, mutating the TRAF1/2/3/5 binding sites, if anything, slightly aggravated lesions (1093). In prior studies, prolonged shear stress in human EC was shown to increase TRAF3 (but not TRAF2 or TRAF5) expression, presumably by mRNA or protein stabilization since transcription was unchanged. Increasing TRAF3 expression, either by shear stress or by transfection, blocked endothelial CD40 signaling (as manifest by reduced IL-8, MCP-1, and tissue factor production) triggered by CD40L expressed on activated platelets. Interestingly, in EC, TRAF3 did not localize with the CD40 receptor but was instead shown to accumulate in the nucleus and directly block DNA binding by AP-1. In EC, CD40-induced MCP-1 production was shown to be mediated by JNK, ERK1/2, and AP-1 rather than by NF-κB (1836). These observations illustrate the diversity of signaling pathways a single receptor may trigger and how the emphasized pathway may differ from cell to cell.

**5. NF-κB transactivation**

Transactivation involves posttranslational protein modifications of the NF-κB dimer, allowing it to function as a competent transcription factor whose activity is further modulated by other coactivators and corepressors. Phosphorylation of RelA at Ser276 is critical in facilitating RelA binding and acetylation by the coactivator CBP (CREB binding protein or closely related p300) and binding with other coactivators (such as cyclin T1/CDK9) which can direct expression of a more inflammatory subset of NF-κB target genes (623, 1320). Ser276 also vies for attention from the E3 ubiquitin ligase SOCS1 (suppressor of cytokine signaling 1) that targets RelA for degradation and the oncogenic kinase Pim1 (proviral integration site murine leukemia virus) that can maintain RelA active (1307). Several other posttranslational modifications are shown in **Figure 4** (623). Various kinases not generally considered part of the NF-κB “signa-losome” can contribute to activation of nuclear NF-κB, such as endothelial focal adhesion kinase (FAK) (1414). Nitration by peroxynitrite (on RelA tyrosines 66 and 152), or nitrosylation by NO of cysteine 62 of p50, inhibits NF-κB signaling by causing dissociation from DNA and removal from the nucleus by IkBα (623).

NF-κB complexed with CBP/p300 displaces inhibitory histone deacetylases (e.g., HDAC1–3) and promotes activity of histone acetyltransferases (HATs). Histone acetylation leads to transcriptional activation of target genes by relaxing the winding of DNA around histones, making the promoter more accessible to the RNA polymerase II complex. Interestingly, cigarette smoke extract powerfully inactivates HDAC enzymes to promote inflammation while GR (glucocorticoid receptor)-bound cortisol recruits HDAC2 to inflammatory gene promoters to repress inflammatory gene expression (623). Additionally, cortisol-GR exerts powerful anti-inflammatory effects by acting at glucocorticoid-responsive elements (GREs) as a positive transcription factor to various anti-inflammatory proteins (such as annexin A1) (1407) and by binding to other adaptor proteins, in particular GR interacting protein 1 (GRIP1) which binds and acts as a repressor to most NF-κB and AP-1 responsive proinflammatory genes (308, 1482). Estrogen also induces annexin A1 with diminished adhesion to endothelium by estrogen-treated neutrophils (1267). In cholesterol-fed rabbits and ApoE-deficient mice, glucocorticoid treatment decreased plaque size (81, 549), while no effect was seen in ovariec-tomized monkeys without estrogen replacement (11). However, medroxyprogesterone blocked the protective effective of estrogen treatment in these animals. Macrophage-specific deletion of GR did not affect lesion size in LDLR-deficient mice but resulted in lesions with less calcification (1437).

Silent mating type information regulation 2 (Sir2) homolog 1 (SIRT1) is an anti-inflammatory deacetylase that can inactivate NF-κB by removing acetyl groups. Endothelial-spe-
Table 2. NF-κB activation of nuclear translocation by representative members of the TNFR superfamily of receptors

<table>
<thead>
<tr>
<th>Downstream Signaling Proteins (Transmembrane Receptor Adaptors)</th>
<th>Receptors (Conformation, Extracellular or Transmembrane Adaptors)</th>
<th>TLR4 (dimer, MD2, CD14, LBP, RP105, MD1)</th>
<th>CD40 (trimer)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary intracellular adapters</strong></td>
<td>recruited to the cytoplasmic tail of the ligated receptor. Additional adapters, kinases, and E3 ubiquitin ligases may bind to the activated receptor.</td>
<td></td>
<td></td>
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<tr>
<td>SODD (inhibitory, displaced by TRADD)</td>
<td>TRADD (recruits TRAF2, RIP1)</td>
<td>MyD88 [recruits IRAK4 then IRAK2 and/or IRAK1, Tollip, TRAF6]</td>
<td>TRAF's bind directly to receptor's cytoplasmic tail. Binding sites for TRAF3 and TRAF2 (alternative) and TRAF6 (canonical) overlap causing competitive inhibition of alternative versus canonical signaling.</td>
</tr>
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<td>TRADD (recruits TRAF2, RIP1)</td>
<td>TRUSS (recruits TRAF2, IKK complex)</td>
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<td>TRADD (recruits TRAF2, IKK complex)</td>
<td>MyD88 (see for IL-1R) Tollip (see for IL-1R)</td>
<td>TRAM (binds receptor at a domain separate from MyD88 and recruits TRIF)</td>
<td>In the cytoplasm of resting cells, TRAF3 recruits NIK and TRAF2, TRAF2 recruits cIAP1/2 which links K48 chains to NIK leading to constitutive NIK degradation.</td>
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<td><strong>Activating E3 ubiquitin ligases</strong> (targets, chain type) also serve as E3 targets with self ubiquitination or from another E3</td>
<td><strong>TNFR1 (trimer)</strong> IL-1R (+IL-1RacP) TLR4 (dimer, MD2, CD14, LBP, RP105, MD1) CD40 (trimer)</td>
</tr>
<tr>
<td><strong>IKK activating kinases</strong> (target) activated by ubiquitin scaffold in canonical pathway</td>
<td>TAK1(^c) (IKK2) ASK1 (MAP2K) MEKK1 (IKK2) MEKK3(^a) (IKK1, MKK4/7, MKK3/6) MLK3 (MKK4/7) PKC(\zeta) (IKK2, others) SCF-Skp2 (IkB(\alpha), K63) SCF-(\beta)TrCP (IkB(\alpha), p100, major E3 producing K48 chains) A20 (RIP1, TRAF, NEMO, Tak1, K63 chains. Induced by NF-kB) USP21 (RIP1 (1998)) CYLD (similar to A20. Constitutively active. Particularly important for NEMO, TRAF2, TRAF5, and Bcl-3 K63 chain removal)</td>
</tr>
<tr>
<td><strong>Deactivating K48 E3 ligases and deubiquitinases or ubiquitin editing enzymes (targets)</strong></td>
<td>Pellino (RAK1, K63) Pellino-2 (Bcl10, K63) TRAF6 (TRAF6, Tak1, NEMO, MALT1, RIP1, K63) Pellino (RAK1, K63) TRAF6 (TRAF6, Tak1, NEMO, MALT1, RIP1, K63) Pellino-2 (Bcl10, K63)</td>
</tr>
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General: IL-1RacP (IL-1R activating protein) acts as a heterodimer with IL-1R. Oligomerization appears to be required for activation of several components [such as the complex of 6 MyD88, 4 TRAF4, and 4 TRAF2 that are recruited in that order to the activated TLR/IL-1 receptors] (1048). RIP1 appears not to be uniquely required or is redundant for TNFR1 activation of NF-κB (1976). IKK2 may only phosphorylate S32 on IkB\(\alpha\), leading to its ubiquitination and degradation while IKK1 phosphorylates S36, followed by dissociation from NF-kB without degradation (2028). CD40 can signal through both the alternative and the canonical pathways but alternative predominates and only this pathway is shown. E2 ubiquitin conjugating enzymes are not shown. A role for the E3 ligase ZFP91 was recently shown for CD40 signaling (853).

*MEKK3 bypasses Tak1 activation but requires p62 (which binds ubiquitin chains on TRAF6 or Rip1) to recruit PKC\(\zeta\) and MEKK3 to the signalosome (1277). MEKK3 phosphorylation of IKK1 results in IkB\(\alpha\) phosphorylation on Ser36 by IKK1 (as opposed to Ser32 by IKK2) that leads to dissociation of IkB\(\alpha\) but without ubiquitination and degradation (2028). MEKK3 activation is primarily important in IL-1 signaling. *TRAF2 and TRAF5 each appear to largely compensate for absence of the other in NF-kB signaling but knockout of both does arrest TNFR1 signaling to NF-kB. They may serve primarily as scaffolds for cIAP1/2. In this regard, TRAF2 appears more active (2075). They also recruit Rip1 to the complex for K63 ubiquitination by cIAP1/2. This is thought to direct TNFR1 signaling toward NF-kB signaling rather than toward apoptotic signaling through FADD complex formation. TRAF2 E3 ubiquitin ligase activity may only be relevant for ASK1 activation. In TNFR1 signaling, cIAP1/2 is the main E3 ligase for Rip1, decorating it with K63 ubiquitin chains. LUBAC is thought to bind K63 chains but produce linear chain for NEMO.
cific (2079) and whole body (1680) overexpression of SIRT1 decreased atherosclerosis while decreased SIRT1 expression increased atherosclerosis (1680). Heterozygous deficiency caused greater endothelial NF-κB activity, increased ICAM-1 and VCAM-1 expression, and greater sensitivity to TNF-α (1681). Overexpression of SIRT1 protected endothelial cells from oxLDL induced apoptosis (2079). Importantly, 1-year caloric restriction doubled SIRT1 in mouse aorta compared with ad libitum feeding while 6 mo of high-fat diet decreased SIRT1 by 50% (2079).

6. Canonical NF-κB signaling through the CBM complex

Immune activating receptors such as T-cell receptor (TCR), B-cell receptor (BCR), and activating IgG receptors such as FcγRI, have immunoreceptor tyrosine-based activation motifs (ITAM) domains in the receptor or in associated adapter or coreceptor proteins. ITAM-bearing receptors and adapters lack autocalytic activity. Instead, receptor ligation leads to recruitment and activation of the membrane-bound tyrosine kinase c-Src (or a related family member such as Lck) which phosphorylates the ITAM motif (and other targets) at two closely spaced tyrosines, thereby recruiting the cytoplasmic tyrosine kinase Syk (spleen tyrosine kinase) or a closely related tyrosine kinase (such as ZAP-70 in T-cells) (1224). Binding of Syk leads to steric alteration allowing further activation of Syk through tyrosine phosphorylation by a SFK (Src family kinase). Activated Syk then phosphorylates target tyrosines on many proteins including various scaffolds, allowing downstream inflammatory signaling. Tyrosine phosphorylated Syk itself becomes a scaffold to provide activating binding for a number of signaling molecules including PLCγ, PI3K, and Vav (a GEF for Rac). Orally administered fostamatinib, a recently developed Syk inhibitor, reduced atherosclerosis in LDLR-deficient mice by as much as 58% (767). Both endothelial inflammation and monocyte migration were inhibited.

In T-cell signaling, PI3Kδ recognizes specific scaffold phosphotyrosines produced by Syk and is activated, especially when the costimulatory receptor CD28 is activated simultaneously, binding and activating additional PI3K. PI3Kδ generates membrane-bound PIP₃, required for PLCγ activity, whereupon PLCγ cleaves membrane-bound PIP₂ to generate free IP₃ and membrane-bound DAG. IP₃ opens the IP₃ receptor to release calcium from the ER. Free calcium and DAG together activate conventional and novel PKCs. One of these PKC enzymes can then phosphorylate the inactive cytoplasmic adapter CARD 11 (caspase-recruitment domain-containing adapter protein 11, also known as CARMA1) causing translocation to the membrane, formation of a trimer and recruitment of two copies of the adapter Bcl10 which then bind mucosa-associated lymphoid tissue I (MALT1) as a trimer. MALT1 recruits and activates TRAF6 while also inactivating A20 (1462). NIK may also be activated (142). Active TRAF6 deco-

rates MALT1, Bcl10, NEMO, and itself with K63 ubiquitin chains, leading to TAK1 recruitment, activation of IKK2, IκBα degradation, and translocation of NF-κB. This activation pathway also explains the long observed phenomenon that phorbol esters (which are thought to activate PKCs by mimicking DAG) can activate NF-κB (1616). CARD/CARMA, Bcl10, and MALT1 are together known as the CBM complex.

A number of receptors signal through the CBM complex by way of different CARD adapters. However, all CBM complexes seem to share Bcl10. ApoE-deficient mice with Bcl10 KO had markedly attenuated atherosclerosis (see TABLE 1) (1164).

7. Additional notes on NF-κB signaling

The adapter, MADD (MAPK-activating, death domain-containing protein) can bind TNFR1 after ligand binding and direct signaling through Grb2 and Sos to Ras-Raf-1-ERK1/2 (963). One downstream target of ERK1/2 is RSK1 (ribosomal S6 kinase 1, also known as p90RSK1 or RPS6KA1). RSK1 can phosphorylate IκBβ (on serines 19 and 23), causing subsequent ubiquitination and degradation and delayed but prolonged NF-κB activation (2001). RSK1 activation by way of ERK1/2 and JNK is also mediated by ANG II signaling (705). Another RSK, RSK2, may also be involved NF-κB activation (1401). Still other kinases seem to be involved in TNFR1 signaling. Thus MLK2/3 (mixed lineage kinases 2 or 3, or MAP3K10/11), activated by germline center kinase (GCK, or MAP4K2) appear necessary for TNFR1 signaling to activate JNK (2099).

Both TNFR1 and integrin signaling can activate Bcl-3 (a protein unrelated to Bcl-2 family proteins or Bcl-10). Bcl-3 is an IκB family protein which functions quite differently from other traditional IκB proteins. When active, apparently after K63 ubiquitination, it can bind to inhibitory, DNA-bound p50 or p52 dimers and act as an activating transcription factor. Bcl-3 also binds and activates the Src-family tyrosine kinase Fyn which becomes important for clot retraction through de novo Bcl-3 synthesis stimulated by thrombin signaling in platelets (736, 1841, 1952). Relevance to atherosclerosis appears to be unexplored.

For IL-1R and TLR signaling, the adapter myeloid differentiation primary-response protein-88 (MyD88) plays a role analogous to TRADD for TNFR1 while interleukin-1 receptor-associated kinase (IRAK) proteins are analogous to RIP1. Both MyD88 and IRAK4 deletions strongly reduce atherosclerosis (TABLE 1). TRAF6 is the major E3 ligase for IL-1R and TLR signaling as well as for the ITAM receptors noted above. Oligomerization of TNF family receptor components and adaptors may be key to activation. Ligation of TNFR1,
IL-1R, or TLR triggers hierarchical oligomerization of receptors, adapters, and E3 ligases. For the typical TLR, the transmembrane, question mark-shaped receptor acts as a dimer and recruits six MyD88 adapters which in turn recruit 4 IRAK4 and 4 IRAK2 molecules, resulting in a complex described as a helical signaling tower (1048). There is some question whether IRAK2 is redundant for IRAK1 or whether IRAK2 is even an active kinase. Recent data, however, suggest IRAK2 is particularly important in human monocyte/macrophage signaling and contributes to generation of active NF-κB and, in some way, p38 (527). TRAF6 and Pellino-2 are major E3 ubiquitin ligases recruited to the complex.

IL-1R (and other receptors) can also trigger NF-κB translocation by activation of MEKK3. The scaffolding protein p62 (also known as sequestosome 1) binds to polyubiquitin chains on TRAF6, bringing with it MEKK3 and PKCζ (1277). MEKK3 (being a MAP3K) has been variously reported to activate the IKK complex by phosphorylation of NEMO, IKK1, or IKK2 (1715). MEKK3 can also activate downstream MAP2K and MAPK.

A number of other means of NF-κB activation are known. Intracellular TLR and related receptors which recognize and bind bacterial or viral structural patterns, such as double-stranded RNA, can signal to activate the IKK complex. Of great significance to atherosclerosis is a connection between ER stress (also referred to as the unfolded protein response) and NF-κB signaling (1138). Three transmembrane transducers or sensors extend into the ER lumen and bind various chaperone proteins. Chaperones assist in proper maturation and folding of newly synthesized proteins. When chaperones are unavailable to bind the luminal side of these sensors, due to over-utilization when protein production rates are very high or because of other stresses that interfere with protein folding, the transducers become activated and initiate multiple signaling pathways. One of these sensors is inositol-requiring enzyme 1 (IRE1) which, in the face of ER stress, binds and activates TRAF2 thereby triggering canonical NF-κB signaling. ER stress and subsequent NF-κB activation was seen in endothelial cells exposed to acrolein and a number of other reactive aldehydes (687).

D. ROS in Inflammatory Signaling

1. General ROS signaling mechanisms

H₂O₂ is now generally considered a facilitator or modulator of inflammatory signaling rather than a direct instigator of NF-κB signaling as had previously been thought (1345). Furthermore, its effects are cell and context specific with arterial endothelial cells being generally more resistant to adverse effects than a number of other cell types. H₂O₂ is also a major endothelial-derived hyperpolarizing factor (EDHF) mediating vasodilation by stimulation of a VSMC potassium channel (255, 256). Nevertheless, ROS may promote activation of other pathways that interact with NF-κB, such as the ASK1-JNK pathway as illustrated in FIGURE 5. Reactive nitrogen species (RNS) may refer to NO or peroxynitrite formed from the rapid reaction between NO and superoxide. RNS can react with signaling proteins and have effects ranging from similar to opposite those of ROS.

In recent years, signal transduction by ROS and RNS has come to be recognized most often as the result of specific, discrete, reversible, posttranslational modifications typically involving targeted cysteine sulfhydryl groups. Spontaneous or catalyzed conversions from reduced to oxidized cysteine modify the activity, binding, or signaling properties of numerous redox-sensitive proteins, generally in a context-appropriate and harmonious manner. Indeed, ROS-mediated signaling is beginning to appear more akin to phosphorylative signaling, involving a vast array of proteins in normal physiology (1006). To this should be added effects of H₂S (hydrogen sulfide) which also targets cysteine sulfhydryl groups (see sect. IVF). Thus redox-sensitive signaling and ROS-induced protein modification may be viewed as fundamental adaptations allowing cells to capitalize on an environment that inevitably includes ROS. This is in stark contrast to the view that ROS and RNS are demons to be squelched at all cost. Indeed, modest, controlled ROS production is required for numerous vital signaling pathways which include activation, cell survival, proliferation, stress adaptation, cell motility, vasodilation, and angiogenesis.

Progressively higher levels of ROS do lead to apoptosis and eventually necrosis, but even these responses can be viewed as physiological and adaptive in many contexts such as phagocyte release of ROS to induce cell death of virally infected, malignant, or otherwise irreparably damaged cells. Indeed, there are numerous examples of mutations or knockouts which are resistant to ROS-induced apoptosis that also promote cancer, suggesting a delicate balance between the anti- and proapoptotic effects of ROS. A few examples of proinflammatory redox mechanisms are illustrated in FIGURE 5 and described below.

An early recognized effect of ROS was the inactivation of various enzymes that have a vulnerable, catalytically important cysteine in their active site. For example, glutathione peroxidase (GPx) is inactivated by ROS through oxidation of a key cysteine thiol (-SH) group, a reaction that is reversed by incubation with reduced glutathione (GSH) (159). Later, it became clear that such reactions represent a vast array of cysteine or thiol “switches” that are sensitive to the redox potential of the cell (1398, 1517, 1997). Susceptible cysteines are those that easily lose their thiol hydrogen (i.e., with low pKₐ) due to surrounding side chains that stabilize the thiolate anion (protein-Cys-S⁻). This nucleo-
philic thiolate can react with H₂O₂ to produce the relatively unstable sulfenic acid (protein-Cys-SOH). Addition of nitric oxide (NO) or other RNS to thiolate can also yield sulfenic acid after hydrolysis. Further oxidation to sulfinic acid (Cys-SOOH) and finally sulfonic acid (Cys-SO₃H) generally proceeds more slowly. Sulfonic acid derivatives are generally regarded as irreducible by physiological systems and lead to degradation of the protein. Sulfinic groups on peroxiredoxin (Prdx) peroxidases can be reduced by the recently identified sulfiredoxin (Srx) (269).

For many proteins, oxidation to sulfinic or sulfonic acids can cause protein dysfunction and prompt degradation. However, sulfenic acid can react with Cys-SH to form internal or protein-protein disulfide bonds or disulfide bonds with the key antioxidant tripeptide glutathione. Under ox-
idizing conditions, two glutathione moieties can spontaneously form a disulfide bond, yielding oxidized glutathione (GSSG). Each of these sulfur oxidations yields electrons that help quench ROS radicals and protect cysteine sulfhydryl groups from further oxidation.

Protein glutathionylation may occur spontaneously or may be catalyzed by a number of GST (glutathione-S-transferase) enzymes. Kinetic considerations suggest that, in vivo, many of these reactions are probably catalyzed. In addition, glutathione conjugation by GST enzymes promotes metabolism and elimination of a number of electrophilic compounds including reactive aldehydes or “conjugated carbonyls” (a carbonyl carbon or nitrogen group with a double bond between the α and β carbons) (478).

Disulfide bonds generally protect vulnerable protein cysteines from further oxidation but may simultaneously alter function or inhibit enzymatic activity (1951). GSSG and protein-glutathione disulfides can be reduced by the Grx/GTR (glutaredoxin/glutathione reductase) system while intraprotein and protein-protein disulfide bonds as well as sulfenic acid residues can be reduced by the thioredoxin/thioredoxin reductase (Trx/TrxR) systems. Some intraprotein disulfide bonds form and are reduced reversibly and spontaneously based on the cell’s redox potential or H2O2 concentration.

An example of an important nonenzymatic sulfur switch is the recently identified reaction between eNOS and GSSG leading to reversible glutathionylation of two highly conserved cysteines which causes eNOS uncoupling (causing eNOS to produce superoxide rather than NO) (290). This mechanism could conceivably recruit eNOS for extra superoxide production when needed while simultaneously protecting the enzyme from undue oxidative damage.

In relation to general mechanisms of inflammation, among the most important consequences of increased ROS or RNS is the reversible inactivation of protein phosphatases. Most protein tyrosine phosphatases (PTPs) are inhibited by ROS by formation of intraprotein disulfide bonds between cysteine at the reactive site and a nearby “back door” cysteine. Alteration of zinc binding sites may occur or an amide may be formed with the reactive cysteine in some cases (208). Also inactivated by cysteine oxidation are PTEN and SHIP (Src homology 2 domain-containing inositol phosphatase) enzymes. These are important phosphatases that primarily inhibit activating pathways by cleaving the 3-phosphate of PIP3. While the widely expressed serine/threonine protein phosphatase PP2A can also be inactivated by sufficiently high ROS (208), in some circumstances ROS actually increases PP2A activity followed by dephosphorylation and inhibition of several proteins that control entry into the cell cycle and cell proliferation (327).

Newer understanding of peroxidase enzymes, particularly Prdx1/2, demonstrates considerable complexity in the ability of the cell to fine-tune ROS-related signaling. Prdx1/2 and Gpx isoforms are widely distributed throughout the cell and have high affinity for H2O2. They are thought to act primarily at low concentrations of H2O2 with Prdx1/2 particularly involved in maintaining PTEN active when ROS is low. At lower H2O2, Prdx1/2 act primarily as dimers. Cysteine 50 or 51 is the catalytically active site and is readily oxidized to sulfenic acid by H2O2. Sulfenic cysteine 50/51 can be reduced by the Trx/TrxR system while sulfenic cysteines are reduced by Srx. Prdx1/2 dimers physically interact with and maintain PTEN activity while also neutralizing low concentrations of H2O2. A similar interaction apparently helps maintain JNK in an active state at low levels of ROS (1294). With higher ROS, Prdx1/2 is rapidly oxidized and inactivated as a peroxidase. At the same time, Prdx1/2 dimers aggregate to form dimers that can act as chaperones for other cell signaling proteins (such as several involved in cell division or apoptosis) (269). The higher order oligomers allow increasing ROS to inhibit PTEN so that PIP3 signaling can proceed while also releasing JNK to be activated by upstream signaling (1294). Furthermore, Prdx1/2 can be variously phosphorylated, nitrated, acetylated, or glutathionylated in ways that can inhibit or otherwise alter activity or affect oligomer formation (269). Catalase, which is largely restricted to intracellular peroxisomes, has greater enzymatic capacity but lower affinity for H2O2. It is thought to become more important at higher H2O2 concentrations (1294). Prdx1 KO mice have a 15% reduction in lifespan. Prdx6 KO mice are also viable and do not display obvious gross pathology, but are more sensitive to certain exogenous sources of oxidative stress, such as hyperxemia.

2. MAPK signaling activation by ROS

With regard to MAPK pathways, inactivation of PTPs prolongs MAPK activation, particularly JNK, with increased subsequent susceptibility to apoptosis (876). Ras activation is enhanced by glutathionylation of Cys118 in the presence of H2O2 and inhibited in a reducing milieu (9). ROS-stimulated disulfide bonding between caspase-9 and apoptotic protease-activating factor 1 (ApaF-1) can promote initiation of the apoptotic cascade (1398).

One of the major activators of both the JNK and p38 MAPKs is ASK1, which is held inactive when redox potential is low by a physical interaction with reduced Trx as well as a 14-3-3 protein as illustrated in Figure 5. In this depiction, activation of TNFR1 provides a complex of TRADD, TRAF2, RIP, ASK1-interacting protein 1 (AIP1), and a K63 ubiquitin scaffold. These all work together to activate ASK1. The adapter protein AIP1 has an overall protective effect in atherogenesis and can suppress both JNK and NF-kB signaling in endothelial cells (793). In an oxidizing environment, both Trx and 14-3-3 dissociate from ASK1; Trx because of internal disulfide bond formation and 14-3-3 because of PKD1 phosphorylation stimulated by ROS. ROS also promotes ASK1 dimerization by disulfide bonding and oxidation of ASK1 Cys250 (prevented by Trx bind-
Upon exposure to ROS, GST becomes activated directly (Sterile 20/oxidant stress response kinase 1) or GSTP1. As depicted in FIGURE 5, ROS-mediated activation of SOK1 (Sterile 20/oxidant stress response kinase 1) may further contribute to ASK1 activation through 14-3-3 phosphorylation (1314). Additionally, ROS dissociates SENP1 (sentrin/SUMO-specific protease 1) from Trx, allowing its nuclear translocation. Various ROS-generating stimuli enhance this “atypical” activation including reoxygenation after hypoxia (822), elevated glucose (which was inhibited by antioxidants α-lipoic acid or N-acetylcysteine) (2025), thrombin stimulation (145), and ANG II (2003). The IκBα phosphorylation on tyrosine 42 induced by elevated glucose was redundant and only apparent after inhibition of the p26 proteasome. Active c-Src can also enhance IKK2 activation by phosphorylation of Tyr188 and Tyr199, near the activation loop (795). ROS inactivation of phosphatases could also enhance Src and Syk family tyrosine kinase activation of CBM-mediated signaling and NIK (1164).

Several additional discrete redox switches are depicted in FIGURE 5. Type I PKA can be activated by ROS by disulfide dimerization of the regulatory subunits, yielding the active PKA subunit (PKAc) (206) and phosphorylation of p65/RelA Ser276 (842). Dynnein light chain 8 (LC8) binds IκBα under reducing conditions but is removed by ROS through internal disulfide formation. Reduced LC8 is then generated by the disulfide reductase thioredoxin-related protein 14 (TRP14) (870). The deubiquitinase Cezanne actively removes both K63 ubiquitin chains from RIP1 and K-48 ubiquitins from IκBα, thereby increasing IκBα and retention of inactive NF-κB in the cytoplasm. Cezanne activity is inhibited by ROS (476).

4. The isoform p66Shc promotes mitochondrial superoxide production, “hyperglycemic memory,” and aging

Src homology collagen-like (Shc) is a versatile adapter protein that binds phosphotyrosines on the cytoplasmic tail of receptor tyrosine kinases, other tyrosine phosphorylated receptors, and various adapters. It is the target of various kinases as well. Three Shc isoforms are produced by alternate initiation sites. Shc isoforms p46 and p52 can function as

Cysteine 62 of the p50 subunit of NF-κB must be reduced to allow efficient DNA binding but the oxidizing environment of the cytoplasm after cytokine activation ensures its oxidation. However, upon entry into the nucleus, p50 encounters Trx complexed with APE1/Ref1 (AP endonuclease 1/redox factor 1) which quickly reduces p50 Cys62, a mechanism of activation shared by other transcription factors (623). Interestingly, both nuclear localization of Trx and APE1/Ref1 and transcriptional activation of APE1/Ref1 are stimulated by ROS, allowing harmonious nuclear reduction of target transcription factors in the face of cytosolic redox stress (139).

Tyrosine kinase Src and Syk families appear to be activated by ROS, but inactivation of opposing phosphatases may be a major mechanism. Indeed, experimental “activation” of Src or Syk is often accomplished by peroxovanate, which essentially inhibits tyrosine phosphatases by oxidizing their active site cysteine (809), suggesting a substantial degree of constitutive activity for Src and Syk enzymes. Thus an increase in ROS in cells might allow Src and Syk activity to become manifest in various ways that are particularly relevant for NF-κB signaling (841).

TNFR1 is said to form a complex that includes constitutively active c-Src, PI3K, and JAK2 that are further activated upon TNFα binding (1418). Both Src and Syk have been reported to phosphorylate IκBα on tyrosine 42 which leads to nondegradative dissociation from the NF-κB dimer, allowing its nuclear translocation. Various ROS-generating stimuli enhance this “atypical” activation including reoxygenation after hypoxia (822), elevated glucose (which was inhibited by antioxidants α-lipoic acid or N-acetylcysteine) (2025), thrombin stimulation (145), and ANG II (2003). The IκBα phosphorylation on tyrosine 42 induced by elevated glucose was redundant and only apparent after inhibition of the p26 proteasome. Active c-Src can also enhance IKK2 activation by phosphorylation of Tyr188 and Tyr199, near the activation loop (795). ROS inhibition of phosphatases could also enhance Src and Syk family tyrosine kinase activation of CBM-mediated signaling and NIK (1164).

3. ROS effects on NF-κB signaling

Many older reviews depict NF-κB being activated directly by ROS. In reality, a number of discrete systems can lead to activation or inhibition depending on the stimulus and context (208, 1345). At physiological concentrations of ROS NF-κB signaling is generally stimulated, while with very high ROS (such as exposure to activated phagocytes), NF-κB signaling is inhibited. Cys-179 of IKK2 provides an interesting case in point. Exogenous addition of H2O2 led to glutathionylation of Cys-179 of IKK2 causing reversible inactivation (1478). Nitrosylation by RNS of this cysteine residue also inhibits IKK2 as can adducts of several physiologically produced electrophiles including 4-NHE, the biologically produced electrophiles including 4-NHE, the

In nonstressed cells, JNK and TRAF2 are held inactive, complexed with glutathione-S-transferase π1 (GSTπ or GSTP1). Upon exposure to ROS, GSTπ dissociates from JNK and TRAF2 and forms GSTπ oligomers (1997), freeing JNK to activate c-Jun and allowing TNFα1 signaling (see FIGURES 3 AND 4). GSTπ also functions to actively glutathionylate target proteins when activated by ROS, nitrosative stress (1804), and tyrosine phosphorylation by EGFR (1340). Interestingly, arsenic appears to require glutathionylation for its detoxification. In a Korean study, the combination of an inactivating variant (Ileu105Val) of GSTπ with high exposure to arsenic in well water was associated with a sixfold increase in the risk of carotid atherosclerosis (1922).
binds the adapter factor associated with neutral sphingomyelinase domain (DD), a neutral sphingomyelinase domain (NSD) which TNFR1 contains, in addition to the more familiar death domains. Promotion of apoptosis through p66Shc occurs by superoxide production. Transport to mitochondria and activation of p66Shc in the setting of diabetes appears to be mediated through phosphorylation by PKCβII (1377, 1378, 1398, 1801) but also by electrophiles such as acrolein. An increase in glucose in the medium bathing endothelial cells results in persistent activation of PKCβII and p66Shc with increased mitochondrial ROS production persisting even after glucose levels are reduced (1377). Knockdown of p66Shc results in the loss of the so-called endothelial “hyperglycemic memory” (1377). Various antioxidant defenses, including manganese superoxide dismutase and heme oxygenase, are also increased with knockdown of p66Shc (2106). Mice deficient in p66Shc live 30% longer and do not display increases in endothelial iNOS, superoxide production, or nitrotyrosine accumulation with aging as do wild-type mice (536). Furthermore, inactivation of p66Shc by mutating the NH2-terminal CH domain unique to p66Shc (with no effect on p46 and p52 isoform transcription) greatly reduces atherosclerosis in mice (see Table 2). Indeed, p66Shc seems to signal cells to age generally (1809) and even promotes insulrin resistance (1457). Interestingly, p66Shc expression is specifically inhibited by SIRT1 (2106). Nevertheless, p66Shc deficiency can lead to aggressive metastatic cancer through resistance to apoptosis signals (1102, 1990), yet another example of the need for balance in apoptotic signaling.

E. Ceramide Promotes Endothelial Activation

Ceramide often acts to enhance MAPK activation and other activating pathways. The diverse functions of ceramide are explained in part by the remarkable ability of ceramide to promote coalescence of many small lipid rafts into large, stable signaling platforms (671). Thus ceramide might be considered a relatively low-affinity, low-specificity activator. Ceramide is converted by ceramidase to sphingosine which may also be considered proinflammatory like ceramide. Phosphorylation of sphingosine by sphingosine kinase yields sphingosine-1-phosphate (S1P), a lipid signaling molecule that acts through high-affinity GPCRs with generally anti-inflammatory effects that quite effectively oppose ceramide, even though intracellular levels of S1P are ~3,000-fold lower than ceramide (711).

TFR1 contains, in addition to the more familiar death domain (DD), a neutral sphingomyelinase domain (NSD) which binds the adapter factor associated with neutral sphingomyelinase activation (FAN). FAN recruits other adapters, receptor for activated C kinase 1 (RACK1) and embryonic ectoderm development (EED), which finally recruits neutral sphingomyelinase, an important source of ceramide on the inner leaflet of the cell membrane (1228, 1415). Rapid membrane production of ceramide can be prompted by numerous additional receptors and activating signals. Possibly through redox signaling, these receptors typically activate acid sphingomyelase by a process involving translocation of quiescent acid sphingomyelinase in cytoplasmic vesicles to the outer wall of the cell membrane where the active enzyme cleaves sphingomyelin (671, 1408). Assembly of dispersed signaling proteins such as receptors and NOX2 components (p47phox, NOX2 catalytic subunits) is then facilitated by ceramide-induced fusion of small lipid rafts into a functional, large, signaling platform.

The rate-limiting enzyme for de novo ceramide synthesis is serine-palmitoyl-CoA transferase. Palmitate (the 16-carbon saturated fatty acid, designated C16:0) is the preferred precursor to ceramide (as opposed to the unsaturated fatty acid oleate, for example, which is generally incorporated into triglyceride) (839, 1574). Inhibition of serine-palmitoyl-CoA transferase by the fungal product myriocin decreased atherosclerosis substantially in hyperlipidemic mice (774, 1384) and even promoted regression (1385). Ceramide can be “inactivated” by several pathways including deacylation to sphingosine by ceramidase or by further acylation by O-acetyl-ceramide synthase, also known as lysophosphatidyl 3 (LYPLA3). LYPLA3 deficiency promoted atherosclerosis and sensitized macrophages to apoptosis (1758) (see Table 1).

Interestingly, palmitate can activate JNK by way of mixed lineage kinase 3 (MLK3). MLK3 activation was due to redundant signaling from multiple activated PKC isoforms (PKCα, PKCδ, PKCε, and PKCζ), thought to be activated by DAG and ceramide. Palmitate feeding, as well as obesity due to high-fat diet, have been reported to cause increased endothelial superoxide production dependent on TLR4, BMP4, and upregulation of NOX4, all promoting NF-κB signaling (1131). Release of PKCζ from the inhibitory adapter protein 14-3-3ε seems also to be directly promoted by ceramide, allowing PKCζ to phosphorylate and inactivate the predominantly anti-inflammatory PKB and ERK5 (535).

F. Modulation of Signaling by Electrophiles

The resilience and resourcefulness of cells in adapting to oxidative stress is perhaps nowhere better illustrated than the newly emerging understanding of electrophilic compounds as signaling molecules (208, 659, 660, 1517). Frequently encountered electrophiles include by-products of polyunsaturated fatty acid peroxidation such as malondialdehyde, 4-hydroxy-2-nonenal (4-HNE) from omega-6 fatty acids, 4-hydroxy-2-hexenal (4-HHE) from omega-3 fatty acids, 4-oxo-2-nonenal (4-ONE), acrolein (also in cigarette...
smoke), nitro-alkene derivatives of fatty acids (NO₂-oleate, etc.), prostaglandin by-products such as 15d-PGJ₂, and numerous natural products (isothiocyanate, cinnamaldehyde, etc.). Once considered merely toxic byproducts of cell damage, these reactive electrophiles usually modulate signaling by targeting cysteine thiolute or sulfenic acid groups, but their effects are often distinct from H₂O₂ or other ROS. For example, some aldehydes, such as malondialdehyde, primarily target lysine. Histidine residues may also be modified in some instances.

High doses of several of the above-listed electrophiles (e.g., acrolein) can cause extensive protein alklylation leading to apoptosis or necrosis. Somewhat lower doses can irreversibly deactivate key antioxidant enzymes such as Trx and initiate inflammatory signaling (624). ER stress is promoted by degradation of alkylated proteins by the p20 proteosome (which recognizes misfolded proteins with external lipophilic groups) and degradation of ubiquitinated proteins by the p26 proteosome (659, 687). Some electrophilic adducts can cause proteosomal degradation of GTP cyclohydrolase I (the rate-limiting enzyme for synthesis of the essential eNOS cofactor tetrahydrobiopterin) as well as HSP90, causing eNOS uncoupling and increased superoxide production (1956). Mitochondrial superoxide production may be increased, possibly by uncoupling through direct sulfhydryl modifications of cytochrome c (660, 979) or activation and nuclear transport of p66Shc (1780). Finally, reactive electrophiles can lead to acute depletion of GSH and inactivation of Trx. Some of these effects may be physiological responses promoting greater ROS generation when called for by cellular activation.

Although cytotoxic at higher doses, electrophiles such as 4-HNE form reversible adducts with various signaling proteins suggesting a broader role as ROS sensors and adapters (1973). Ischemic preconditioning, resulting in subsequent protection from ischemic insult, depends in part on electrophilic modifications of signaling proteins (1268). Nitro-fatty acids seem to have a number of potent anti-inflammatory properties such as direct covalent modification and inhibition of IKK2 (660). Subcutaneous administration of nitro-oleate was recently shown to potently inhibit atheroma formation in ApoE-deficient mice (1518). It is tempting to speculate that the surprising anti-atherosclerotic effects of so-called “conjugated linoleic acid” might be mediated by similar mechanisms (1166). 15d-PGJ₂ can covalently bind and activate the strongly anti-inflammatory PPARγ. It also binds and inhibits IKK1 and IKK2 as well as each of the NF-κB dimers (423).

One study found a surprising increase in longevity after KO of a unique mitochondrial GST that targets 4-HNE for glutathionylation and elimination (1636). The modestly increased but physiological levels of 4-HNE that accumulated in the GST knockout mice induced an array of protective antioxidant genes, illustrating the important balance between pro-oxidant and antioxidant forces (208). Hitherto, effects of genetic manipulation of GST enzymes have apparently not been studied with regards to atherosclerosis (738).

In addition to its role in converting glucose to sorbitol, aldose reductase (AR) metabolizes reactive aldehydes formed during oxidative stress. AR was recently found to be activated by the oxidation of Cys298 to sulfenic acid in relatively mild oxidizing conditions. With more severe oxidative stress, AR cysteine298 is glutathionylated by GSTπ. While this modification inactivates AR, it allows later reduction by Grx, thus preserving AR from more severe oxidation and subsequent degradation (1951). Knockout of AR increased atherosclerosis in ApoE-deficient mice (see TABLE 4), suggesting a need for balanced exposure to reactive electrophilic aldehydes.

G. Inflammatory GPCR Signaling as Exemplified by the ANG II Receptor, AT1R

In addition to blood pressure elevation, ANG II directly promotes endothelial activation and inflammation of the arterial wall. ANG II signaling leads to decreased eNOS activity, increased vascular wall ROS production, expression of endothelial adhesion molecules and chemokines, VSMC proliferation, vascular wall remodeling, and atherosclerotic lesions and is prothrombotic (1556). Genetic manipulations of the renin-angiotensin system consistently demonstrateatherogenic effects (see TABLE 1). Chronic ANG II infusion by implanted osmotic minipumps dramatically increased atherosclerosis in hyperlipidemic mice and led to development of aortic aneurysms, effects entirely eliminated by KO of the AT1R (263). Effects were predominantly due to arterial wall expression of AT1R, but hematopoietic expression of AT1R also affected atherosclerosis if arterial wall AT1R was present (263, 563). Blood pressure was clearly affected by these manipulations although not strongly correlated with plaque size. In a study controlling for blood pressure effects, the blood pressure of control ApoE KO mice was matched to the blood pressure of AT1R/ApoE double-deficient mice using two different blood pressure medications. Hydralazine, a vasodilator with no effect on AT1R signaling, had no effect on atherosclerosis lesion size. In contrast, irbesartan, an AT1R blocker, reduced atherosclerosis almost to the same extent as AT1R deficiency (1929). It should be noted here that in human hypertension intervention trials, cardiovascular endpoints were reduced primarily in proportion to blood pressure reduction with surprisingly little evidence for additional benefits from ACE inhibitors or ARBs (ANG II receptor blockers) (1822, 1823).

There appear to be interactions between ANG II and lipids in atherogenesis. ANG II (or adrenaline) increased uptake
of LDL into the artery wall of rats (259), while ANG II in plasma and expression of AT1R in the artery wall were markedly increased by hyperlipidemia in mice (384). ANG II content of the aorta was increased together with atherosclerosis in cholesterol-fed monkeys (1220). ANG II can be generated from mast cell chymase in human and animal arteries (75, 1168, 1799). Enzymatic ANG II-generating activity was correlated with LDL levels in human arteries, with more marked increase in atherosclerotic aorta (75, 1831), and was markedly increased in adventitia along with chymase after cholesterol and saturated fat feeding in hamsters (1830). Arterial intimal ANG II increased fivefold with age in non-human primates (1904). Chymase inhibition was recently shown to decrease atherosclerosis in ApoE-deficient mice (187).

1. A brief introduction to GPCR signaling

The 748 human GPCR (683 of the Rhodopsin class, 33 Adhesion, 22 Glutamate, and 10 Frizzled) (952) are all characterized by multiple transmembrane helical domains. Commonly encountered GPCR (all in the Rhodopsin and Frizzled classes) have seven transmembrane domains. In the resting state with no bound extracellular ligand, the intracellular COOH terminus of these GPCR binds a Gα protein with covalently attached GDP. The Gα protein is accompanied by a heterodimeric βγ subunit. Extracellular binding of a ligand to its specific GPCR generates a change in the intracellular configuration of the receptor which triggers the binding of GTP to the bound Gα protein. Visual receptors (rhodopsins) are GPCR with a covalently bound ligand, 11-cis-retinal, which is converted to 11-trans-retinal after absorbing a photon. The shape change results in activation of the receptor and the release of the 11-trans-retinal. Both the activated Gα protein and the accompanying βγ subunits can then participate in subsequent signaling. Heterotrimeric G proteins are all membrane bound with the 16 different Gα proteins being palmitoylated or myristoylated, and the 5 β and 11 γ isoforms being prenylated. Among the commonly encountered Gα are those that stimulate (Gαs) or inhibit (Gαi) AC (adenylate cyclase). The increase in cAMP from Gαs stimulation of AC goes on to activate the serine/threonine kinase PKA (protein kinase A) with its many targets. Additionally, exchange protein activated by cAMP (EPAC) is a GEF, activated by cAMP, for the G protein Rap1. GTP-bound Rap1 can activate PLCε to produce IP3 and DAG.

Gαi activation is often anti-inflammatory (with PKA having several inhibitory targets, for example, on Raf1). The Gαq proteins are typically pro-inflammatory, both by inhibiting AC and by sometimes stimulating PLCβ (phospholipase Cβ). They can also stimulate PI3Kγ. The Gαq11 isoforms strongly stimulate PLCβ. PLCβ releases DAG and IP3 from PIP2 with IP3 acting on IP3R to release calcium and calcium and DAG promoting activation of classical PKC isoforms. Gα12/13 can activate several RhoGEFs including LARG (leukemia associated RhoGEF), p115RhoGEF, and PDZ-RhoGEF. The βγ subunits released by activated GPCR can, in turn, contribute to activation of PLCβ and PI3K, and have a number of other targets. G protein receptor kinases (GRKs) phosphorylate the activated GPCR to provide binding sites for various adaptor proteins, such as β-arrestins. Binding of β-arrestins causes arrest of G protein signaling and direction to clathrin-coated pits for endosomal removal of the GPCR from the cell surface. However, β-arrestins can also serve as docking proteins for entirely different downstream effectors potentially leading to a complete shift in the initial transduction activities of the GPCR.

2. ANG II signaling through AT1R: the basics

ANG II signaling through AT1R results in a host of proinflammatory responses including a burst of ROS production through NOX1/2 (with NOX1 primarily in VSMC), activation of NF-κB in endothelial cells with increased expression of E-selectin, VCAM-1, ICAM-1, MCP-1, and promotion of monocyte adhesion, as well as potentially proinflammatory, proatherogenic effects on macrophages and VSMC (263). These inflammatory effects are essentially all mediated by signaling apart from arterial contraction.

Initially, ANG II binding to AT1R causes GTP exchange for GDP on bound Gαq, GTP-Gαq activates PLCβ with release of DAG and IP3. The βγ subunits also contribute to PLCβ activation. PI3Kγ is activated by βγ subunits as well. IP3 binds the IP3R to release calcium from the ER. In VSMC, calcium-CalM can then directly promote contraction by binding myosin light-chain kinase (MLCK). However, little contraction occurs without also inhibiting myosin light-chain phosphatase (MLCP). This is accomplished when GTP-bound RhoA (generated as described below) binds and activates one of its major effectors, ROCK1 or ROCK2 (Rho-associated, coiled-coil containing protein kinase 1 or 2) which inhibits MLCP by phosphorylating the subunit MYPT1 (myosin phosphatase targeting subunit 1) (670). Furthermore, ROCK1/2 can directly phosphorylate myosin light chain to stimulate contraction while also stimulating MLCK. Calcium-CalM would also be expected to enhance eNOS signaling in endothelial cells.

Calcium-CalM also activates calcineurin which is a serine/threonine phosphatase that dephosphorylates the nuclear factor activated by T-cells (NFAT) family of transcription factors, NFATc1–4. Dephosphorylation of NFAT allows translocation to the nucleus. Interestingly, nuclear GSK-3β and JNK phosphorylate NFAT to stimulate transport back out of the nucleus. The NFAT transcription factors mediate pro-inflammatory gene transcription in a number of cell types. The anti-inflammatory, anti-rejection effects of cyclosporin A and tacrolimus are due to their inhibition of calcineurin. Calcineurin is also activated by ANG II signaling. Calcineurin in some way appears to directly stabilize MCP-1 without increasing transcription (1542). Other po-
tential effects of NFAT on atherosclerosis remain poorly understood (1310). Hereafter, AT1R signaling becomes far more complex.

3. Inflammatory signaling through AT1R is largely mediated by PYK2

Early studies clearly identified activation of ERK1/2 as a downstream consequence of certain ligated GPCR, but the mechanism remained obscure. The effect was seen primarily in Gαq and Gα1-coupled GPCR (including AT1R) and required activation of PKC (usually atypical isoforms such as δ and ε) and/or increased intracellular calcium and ROS, all potentially attributable to the PLCβ activated by these receptors. Merely increased calcium and ROS, seen with activation of many receptors and ion channels, can also activate ERK1/2.

The tyrosine kinase PYK2 (proline-rich tyrosine kinase 2, also known as FAK2 or PTK2B for protein tyrosine kinase 2, beta) was found to be the primary mediator of these effects in many cells (426, 1011, 1520). While PYK2 is highly analogous to FAK, it is primarily cytosolic while FAK is mainly associated with integrin signaling and is activated by different signals. PYK2 is highly expressed in leukocytes and brain, but is also found in endothelial cells and VSMC. While initially thought to be directly activated by calcium, subsequent studies found no calcium binding sites on the enzyme, although some data suggest a potential role for calcium-CaM binding (929). Most clearly, however, PYK2 is activated by PKC (particularly PKCδ) and calcium/CaM-dependent protein kinase II (CaMKII) (616, 617, 1496, 1497, 1980). Both the calcium, released from ER by IP3 acting on its receptor, and the DAG to activate atypical PKC are supplied by PLCβ activated by AT1R. In addition, an increase in ROS leads to Thr286 phosphorylation of CaMKII which enhances its activity and accounts for the association of ROS with PYK2 activation and downstream signaling (189). The precise mechanism whereby PKC and CaMKII, both serine/threonine kinases, stimulate the activation of PYK2 remains unclear, but may be related to relocation of inactive PYK2 bound to paxillin (associated with integrins and microfibers) to a more vulnerable position or conformation (342, 1673).

Stimulated PYK2, acting as a homodimer or higher order oligomer, trans-phosphorylates Tyr420 on adjacent PYK2 molecules. This provides a binding site for Src or another SFK which phosphorylates Tyr579 and Tyr580 for full activation of PYK2 (1487). Once tyrosine phosphorylated, PYK2 acts not only as a tyrosine kinase but as a scaffold for other signaling molecules which possess domains that recognize its phosphotyrosine motifs. As will be discussed below, similar signaling occurs with FAK. Thus the adapter p130CAS (p130 Crk-associated substrate), which also figures prominently in FAK signaling, was one of the first identified PYK2 binding partners. Subsequent tyrosine phosphorylation of p130CAS by PYK2 leads to binding and activation of a number of downstream effectors including PAK and JNK (158, 1980). Direct binding of Grb2-Sos to PYK2, Shc, or p130CAS provides a means to activate Ras and the ERK1/2 cascade (1496), thus solving the riddle of ERK activation. PI3K can also bind phosphotyrosines on p130CAS and is activated, producing PI3 for PDK1, PKB, Ras, and NOX activation (1496).

There are a number of other PYK2 targets. PYK2 activates GSK-3β by tyrosine phosphorylation (1813). Additionally, the PYK2-Src complex can apparently activate ADAM17 with release of HB-EGF and transactivation of EGFR signaling, providing an additional source of PI3K and Grb2-Sos-Ras activation (616, 617) and a sustained release of ROS (805). Other binding partners of PYK2 are Vav1 (in leukocytes) (586) and Vav2 (in endothelium) (890). Vav is the sixth letter of the Hebrew alphabet and was chosen as the name for Vav1 since it was the sixth oncogene identified in the laboratory of its Jewish discoverer. The Vav proteins are GEFs for Rac1 or RhoA. They therefore provide GTP-bound Rac1 for NOX1/2 activation.

In VSMC stimulated by ANG II, PYK2 directly binds and activates JAK2 (539), which then stimulates ArhGEl by tyrosine phosphorylation to activate RhoA with subsequent smooth muscle contraction mediated by ROCK1/2 (670). STAT3 may also be activated by JAK2 (1394). A reduction of early atherosclerotic lesions was seen in fat fed mice with endothelial cell-specific STAT3 KO (610). AT1R may also bind JAK proteins directly, and several STAT transcription factors, including STAT1–3, 5, and 6, have reportedly been activated by AT1R signaling (805). ANG II activated PYK2 directly binds and activates PDZ-RhoGEF which serves as another major source of active RhoA in VSMC (2042).

4. Physiologic and atherogenic consequences of PYK2 signaling

The downstream consequences of PYK2 activation are many and varied, and differ by cell type. In endothelial cells, PYK2 signaling stimulated by ANG II was, in part, dependent on NOX2 and resulted in Tyr657 phosphorylation of eNOS, decreasing its activity (1070). Activated ERK1/2 further promotes ROS production by phosphorylating NOX5 and p47phox (1375). CaMKII also activates NOX5 (1376). Nevertheless, endothelial ERK1/2 activated by PYK2 partially overcomes ANG II-induced endothelial eNOS dysfunction by enhancing eNOS activity through phosphorylation of Ser633 (Ser635 in bovine endothelial cells) (1736, 1737, 1994). Endothelial PYK2 also mediates Ras and ERK1/2 activation mediated by LPC (1488), and LPA (lysophosphatidic acid), as well as the bradykinin receptor (426).
PYK2 supports leukocyte adhesion to fibronectin (through integrin β3) (586), macrophage migration (1341), IL-10 production in macrophages in response to zymosan (which signals through the ITAM-containing Dectin receptor with Syk activation) (894), macrophage activation through CD36 binding of oxidized phospholipids (716), and neutrophil degranulation (877). VSMC proliferation induced by PDGF is dependent on PYK2 (1404). ANGII can also activate p38 with downstream activation of MK2. MK2 deletion decreased atherosclerosis, particularly in the setting of continuous ANGII infusion (see Table 1). Not surprisingly, NF-κB signaling through ITAM-containing receptors is synergistic with AT1R or TNFR superfamily receptors in promoting atherosclerosis (834).

PYK2 signaling in the endothelium is clearly atherogenic (890). PYK2 directly activates JNK and supports the ROS production required for JNK activation in ApoE KO mice (890). This study provided further important insights into inflammatory signaling stimulated by PYK2 and JNK. Both JNK and especially ERK1/2 activate the transcription factor Ets-1 by serine/threonine phosphorylation. Ets-1 plays a major role in arterial inflammation (1327), promoting transcription of p21Cip1, E-selectin, P-selectin, VCAM-1, ICAM-1, MCP-1, CXCL1, PAI-1, Sm22α, and fibronectin (516, 792, 890, 1302). In addition, all the components of the NOX2 complex, including NOX2, p22phox, p47phox, and p67phox, were induced by ANG II signaling through Ets-1 (516). Ets-1 expression is itself upregulated by various proinflammatory signals (516), particularly TNF-α-mediated ERK1/2 activation (628). In endothelial cells, JNK was found to be required to induce transcription of the cyclin-dependent kinase inhibitor p21Cip1 and TNF-α (890), possibly through Jun activation together with activated Ets-1.

While originally identified only as mediator of cell cycle arrest, p21Cip1 has more recently been recognized to form complexes with the transcription machinery of many genes and may act as either a corepressor or coactivator (295, 523). Additional support of endothelial atherogenic signaling attributable to p21Cip1 came from a recent study in which the FGFR2 receptor was specifically overexpressed in endothelium with resulting 2.5-fold increase in p21Cip1 and 2.0-fold excess atherosclerosis in ApoE-deficient mice. Several aspects of atherogenic signaling were attenuated by siRNA suppression of p21Cip1 (288). In the case of endothelial PYK2 and JNK signaling, increased p21Cip1 bound the histone acetylase p300 and enhanced its activity, promoting transcription of VCAM-1, MCP-1, and other genes dependent on TNF-α, presumably through NF-κB (890). ANG II-induced p21Cip1 also promoted senescence in VSMC (961). Not surprisingly, atherosclerosis was markedly reduced by PYK2 KO in ApoE-deficient mice (890).

Deficiency of p21Cip1 more modestly diminished atherosclerosis (961).

5. GRK2 and β-arrestin2 mediate activation of NF-κB by AT1R through the CBM complex

Like other GCPR, AT1R is subject to phosphorylation of its cytoplasmic domain by GRKs. GRK2 is known to phosphorylate the AT1R cytoplasmic domain to allow binding of β-arrestin adapter proteins. AT1R can bind either β-arrestin1 or β-arrestin2 with high affinity (1322). Once bound to AT1R, β-arrestin2 in some way promotes activation of CARD10 (also known as CARMA3) followed by assembly of other members of the CBM complex and canonical activation of NF-κB (443). A similar mechanism operates in thrombin signaling (405), a presumed LPC receptor (1708), the IL-8 receptor, and CXCR2 (1148). Additionally, receptor-bound β-arrestin1/2 binds Raf1 and ERK1/2 and promotes their activation while also sequestering active ERK in the cytoplasm (337). In HEK cells, ~40% of the ERK1/2 activity appeared dependent on this β-arrestin2 pathway while the remainder was PKC dependent, presumably through PYK2 signaling (1935). Knockout of β-arrestin2 decreased atherosclerosis modestly in LDLR KO mice but primarily by a decreased accumulation of VSMC (907). Similarly, BMT of hemizygous deficient GRK2 into LDLR KO mice modestly decreased atherosclerosis (1359).

IV. INITIATION OF ATHEROSCLEROSIS: ARTERIAL FLOW PATTERNS ESTABLISH THE DISTRIBUTION OF ATHEROSCLEROTIC LESIONS

Endothelial cells stand as either a barrier to inflammation or its earliest facilitator. Only when activated will endothelial cells express the directing signals that control access of leukocytes to underlying tissues. Arterial flow patterns largely determine whether endothelial cells stand poised for facile inflammatory activation or will resist activating signals. Atherosclerosis develops almost exclusively in areas of slow flow or low shear stress, often with eddy currents, characterized by slow, oscillating (back and forth) flow (283, 1343). Such a pattern is described as “disturbed flow” and even predicts locations where plaques will progress in individual human subjects (1942). Turbulence is not a feature of flow at these sites. Indeed, turbulence, defined as blood flow exceeding the critical Reynolds number, occurs almost nowhere in the normal human circulatory system. The coronary circulation is uniquely predisposed to atherosclerosis (804), probably because of high intraluminal pressure and complete flow cessation and possible reversal during systole, a likely cause of the greatly suppressed eNOS activity observed in coronary compared with aortic endothelial cells (377).
A. Endothelial Activation by Hemodynamic Factors: General Features and Phenotypic Effects

Endothelial activation has been conceptualized as type I (rapid, without new protein synthesis) and type II (requiring new protein synthesis with increased expression of adhesion molecules and cytokines) (1424, 1425). Type I activation has been typified by responses to histamine while type II responses are exemplified by TNF-α signaling. Initial hemodynamic responses and longer term responses to disturbed flow demonstrate aspects of both type I and type II activation.

Multiple transduction mechanisms endow endothelial cells with exquisite sensitivity to changes in shear stress, controlling endothelial cell migration, mitosis, apoptosis, endothelial layer permeability, inflammation (including white cell adhesion), nitric oxide release, and thrombosis (388, 693). The prevailing flow pattern determines the endothelial phenotype. Sophisticated, direct measurement of flow patterns in atherosclerosis-prone versus resistant areas revealed marked differences in endothelial response and function as shown in Figure 6 (375). Endothelial cells exposed to disturbed flow (Figure 6A) develop a proinflammatory phenotype with increased production of ROS, higher cell turnover, increased cell-cell permeability, and excess apoptosis. They express leukocyte adhesion molecules and chemokines. In contrast, the flow pattern in areas protected from atherosclerosis is rapid (high shear stress with laminar flow) and unidirectional, although pulsatile as shown in Figure 6B. Endothelial cells exposed to such flow become quiescent and resistant to inflammatory signals with low intercellular permeability.

Importantly, when endothelial cells cultured without flow are then exposed to onset of flow, or to sudden stepped increases in flow, or especially a change in flow direction, there is transient activation (311). However, after several hours of continued, unidirectional, laminar high shear stress flow, even if pulsatile, these initial proinflammatory responses are suppressed below beginning levels. Thus endothelial cells cultured in a medium without flow are essentially activated to some degree. After prolonged exposure to unidirectional flow (10–12 h, but especially after 24 h), endothelial cells display an anti-inflammatory, antioxidant, antiproliferative phenotype, remaining in a relatively quiescent state but active in production of protective NO and PGI₂ (prostacyclin). They also become elongated, aligning the long axis of their cytoskeleton in the direction of flow. In contrast, where there is repetitive flow reversal, however slight, endothelial cells never suppress their proinflammatory response as evidenced by translocation of NF-κB to the nucleus, increased expression of cell adhesion molecules, secretion of MCP-1 and endothelin, and production of superoxide. NO production is reduced and bioavailability impaired. These endothelial cells do not align with the flow. They have disorganized cytoskeletons and develop other features characteristic of lesion-prone areas including increased apoptosis, frequent mitoses, greater permeability (particularly at sites of mitosis), and a shorter glycocalyx. In addition, endothelial cells exposed to disturbed flow increase production of subendothelial matrix components such as fibronectin, resulting in enhanced inflammatory responses and thickening of the basement membrane (282, 693).

B. Endothelial Activation by Hemodynamic Factors: GPCRs and Ion Channels

1. GPCR, PLCβ, G proteins, and NOX

Within milliseconds after abrupt onset of flow or flow reversal, signals can be measured from GPCR and ion channels with mechanosensory capabilities (Figure 6A). This phase of endothelial activation is similar to the rapid, so-called, “type I” activation of endothelial cells by histamine. Incidentally, the H1 histamine receptor, a GPCR, appears to promote atherosclerosis by increasing vascular permeability for LDL (1515). Calcium channels are also involved in activation of endothelial cells by disturbed flow. Dramatic, rapid increases in intracellular calcium were seen primarily with flow reversal with much lesser effects from stepped increases in unidirectional flow (1188). Several aspects of flow-related type I signaling are depicted in Figure 7.

Several GPCR may be involved in mechanotransduction of endothelial shear stress, but the best characterized is the bradykinin receptor B₂ (BDKRB2) (267). Activation of B₂ receptors by flow is ligand-independent. Mice made deficient in B₂ receptors develop salt-sensitive hypertension (48), but effects on atherosclerosis have apparently not been tested. The signaling described below is illustrated in Figure 7. A number of the steps are redundant with AT1R signaling described above but are presented again here for didactic clarity. Signal transduction from BDKRB2 is mediated by the Gα₁₁/q₁₁ family of heterotrimeric G proteins; hence, it may expected to be inflammatory. Both the active GTP-bound Gα₁₁ and its βγ partner activate PLCβ. In addition, shear-induced release of βγ can also lead to a 10-fold elevation of activated (GTP-bound) Ras within 5 s, considerably faster than many other mechanisms of Ras activation (667). Despite the short duration of the Ras signal (5–10 min) with onset or directional change in flow, it leads to ERK1/2 and JNK activation (after ~30 min) followed by increased surface expression of E-selectin and VCAM-1, together with secretion of MCP-1 after ~90 min (307).

Active PLCβ cleaves IP₃ from membrane-bound PIP₂, leaving DAG to act as a membrane-associated signaling molecule. Liberated IP₃ acts on the IP₃ receptor (IP₃R) to release...
FIGURE 6. Overview of hemodynamic effects on signaling related to atherogenesis. A: characteristics of a highly atherogenic waveform (slow flow with directional change during each cardiac cycle) together with approximate order of resulting signals. B: atheroprotective waveform (rapid flow, high shear stress with pulsatory but unidirectional flow) and associated signaling. There is an initially pro-inflammatory response, similar to early signaling in A, followed by prolonged active suppression of inflammation.
FIGURE 7. Early mechanotransduction of shear stress in endothelial cells, characterized by type I inflammatory signaling through G protein-coupled receptors (GPCR) and ion channels. The downstream signaling from the B2 bradykinin GPCR is typical for many GPCR, but its activation by shear stress without substrate binding is unique to a few GPCR. Shown in yellow boxes are other mechanical shear-stress transducers. COX-1 is a constitutive but low-capacity enzyme. COX-2 is induced by proinflammatory signals generally and by prolonged laminar flow specifically in endothelial cells and has greater overall capacity than COX-1. Endothelial H$_2$O$_2$ has vasodilatory effects on coronary arteries but may have different effects in different arterial beds. AA, arachidonic acid; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; PLD, phospholipase D; ATX, autotaxin; PC, phosphatidylcholine; sGC, soluble guanylate cyclase; Vm, membrane potential (negative inside, thus decrease in Vm means hyperpolarization); wWF, von Willebrand factor; WPb, Weibel-Palade body. Expression of purinergic ATP receptor P2X4 is much greater in veins than arteries; its conductivity for calcium is markedly increased by flow but also requires ATP. Without the effect of angiopoietin 2 (ANGPT2), released from WPb to block the highly anti-inflammatory signaling of ANGPT1 through its receptor Tie2 (not shown), endothelial cells would remain largely unresponsive to subsequent activating signals.
calcium stored in the ER. Cytosolic calcium itself then leads to opening of the ryanodine receptor (RyR) and further opening of IP$_3$R, a process dubbed “calcium-induced calcium release.” Both IP$_3$R and RyR exhibit negative feedback from sufficiently elevated cytosolic calcium, leading to oscillating calcium signals with the possibility of frequency modulation of downstream effectors. Calcium and DAG activate PKCβ, a conventional PKC (which, by definition, requires both calcium and DAG for activation). Calcium may facilitate a conformational change in PKCβ resulting in greater affinity for DAG and membrane phosphatidylserine. Membrane localization then allows phosphorylation by PDK1 followed by two self-phosphorylations and full activation.

Once active, PKCβ acts in endothelial cells largely as an amplifier of inflammatory signaling. Particularly important in this regard is phosphorylation of Raf-1, and more recently recognized, Raf kinase inhibitory protein (RKIP) (921). In its nonphosphorylated form, RKIP binds to and blocks activation of Raf-1, MEK, and ERK, all three kinases of the ERK signaling cassette. Unphosphorylated RKIP also inhibits IKK2. RKIP phosphorylation by PKCβ relieves the inhibition of both Raf-1 and IKK2, allowing activation by upstream signals. Also, once phosphorylated, RKIP moves to bind and inhibit GRK2 which, when active, promotes phosphorylation and binding by adapters such as the β-arrestins noted above or targeted degradation of GPCR in general, presumably the B$_2$ receptor as well. Knockout of PKCβ is strongly antiatherosclerotic. Atherosclerosis reduction is mediated not only by endothelial effects but also by strong inhibition of macrophage function (see Table 3). Interestingly, PKCβ$^{-/-}$ animals are lean, resistant to obesity, and hyperphagic (104), but immunodeficient.

Active PKCβ inhibits eNOS by phosphorylating threonine 495 and increases NOX2 activity by phosphorylating both the p47$^{phox}$ regulatory subunit and the catalytic p22$^{phox}$ subunit (1413). Rac1 (and RhoA) can be activated by p114$^{RhoGEF}$ which, in turn, is activated by βγ subunits from activated GPCR (1312). Vav2 may also possibly activate Rac1 in this pathway.

2. Calcium signaling, ion channels, and Weibel-Palade body exocytosis

Ion channels in the plasma membrane also contribute to flow-mediated intracellular calcium influx. Several of these are shown in Figure 7. Recently, a contribution from the TRPV4 calcium channel was confirmed which appears to function in conjunction with the TRPC1 channel (1101, 1192). Polycystin-2 (PKD2 gene), a calcium channel which acts in cilia together with the mechanosensory protein polycystin-1 (PKD1 gene), also contributes significantly to these inward calcium currents (5, 1287, 1814). While most arterial endothelial cells are not ciliated, cilia are found frequently in atherosclerosis-prone areas, and their formation may be induced by disturbed flow (1847). ATP is released to the extracellular space by endothelial cells in response to flow followed by autocrine stimulation of the purinergic P2X$_4$ calcium channel (encoded by the P2RX4 gene). P2RX4 knockout increased blood pressure, markedly decreased flow-mediated dilation, and impaired arterial remodeling (2007), but effects on atherosclerosis are apparently not known (see sect. VIA for further discussion of purinergic signaling). Rapidly acting, inwardly rectifying potassium channels, Kir2.1 and Kir2.2 and possibly IKCa1 (202) lead to hyperpolarization in response to shear stress and would further enhance inward calcium flux (761). Outwardly rectifying chloride channels (causing depolarization) are activated after the hyperpolarization by potassium channels. It would be of interest to explore the effects on atherosclerosis of targeted deletion of some of these ion channels.

The increase in intracellular calcium stimulates eNOS through calmodulin. Thus the onset of flow results in a sudden initial burst of NO production that is calcium and G protein dependent (through $G_{q/11}$ and $βγ$ as noted above) (1954). In addition, the transient calcium elevation appears to stimulate more prolonged eNOS activation through ERK1/2 (1994). A likely mechanism is through CaMKII activation of PYK2 which can serve as a scaffold to bind and activate the Grb2-Sos pathway for ERK1/2 activation (894, 1736). CaMKII also directly phosphorylates endothelial NOX5 to promote superoxide production (1376). There may also be a mechanical effect of shear stress on glypican and CD44 to assist in caveolin-1 phosphorylation and removal of its suppressive effect on eNOS (1760). Increased NO production is generally considered anti-inflammatory. However, if eNOS is uncoupled from normal NO production by GSSG as noted above, or if sufficient peroxynitrite has oxidized the zinc-thiolate complex of the enzyme or its essential cofactor BH4 (tetrahydrobiopterin), or if there is deficiency of BH4, then normal stimulation of eNOS may yield a net increase in superoxide production (101).

Increased intracellular calcium can stimulate production of superoxide by mitochondria. Nearly all the enzymes of the tricarboxylic acid cycle are upregulated by calcium, linking enhanced ATP generation to muscle contraction in muscle cells. In endothelial cells, however, such calcium signaling is tied to enhanced mitochondrial superoxide production, possibly by inhibition of respiratory chain complex I and III. Furthermore, NO can inhibit complex I (211) while peroxynitrite strongly inhibits complex IV (708). Released ROS may lead to even greater intracellular calcium by stimulating both IP$_3$R and RyR and impairing calcium uptake by sarcoplasmic-endoplasmic reticulum calcium ATPase (SERCA) with potential adverse cellular effects such as promotion of ER stress aggravated by low ER calcium levels (435).
Table 3. Hemodynamic effects on endothelial cells and other early steps in initiation (55 genes tested)

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<thead>
<tr>
<th>Gene</th>
<th>Model</th>
<th>%Δ A</th>
<th>Function, Comment</th>
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<tbody>
<tr>
<td>Sdc-1</td>
<td>Sdc-1−/− apoE−/−</td>
<td>↑ 53%</td>
<td>Sdc-1 (syndecan-1) was also expressed on macrophages but flow-induced protection of endothelium is a likely mechanism (SS8). While protective, Sdc-1 is listed here as it is a major component of mechanotransduction, directly linking the glycolyx to cytoskeletal stress fibers.</td>
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<tr>
<td>PFN1</td>
<td>PFN1+/− LDLR−/−</td>
<td>↓ 61%</td>
<td>PFN1 (profilin 1) is a key molecule for actin microfiber assembly by binding and priming globular actin with ATP in exchange for ADP. Homozygous deficiency is embryolethal by the 2-cell stage. Nevertheless, increased expression appears proinflammatory while heterozygous deficient mice had reduced p38 and ATF2 signaling, decreased VCAM-1 expression, and enhanced eNOS activity. Macrophones in heterozygous mice had reduced CD36-mediated uptake of oxLDL and inflammatory signaling. Fewer macrophones accumulated in lesions (1503). Higher levels are associated with plaque and plaque-prone areas while laminar shear stress lowers PFN1 expression (325). Diabetes and oxysterol increases PFN1 expression through STAT3 signaling (1502).</td>
</tr>
<tr>
<td>PECAM-1</td>
<td>PECAM-1−/− apoE−/−</td>
<td>↓ 41%</td>
<td>siRNA knockdown of PECAM-1 decreased NF-κB and VCAM1 expression in endothelial cells exposed to disturbed flow (728).</td>
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<td>PECAM-1−/− LDLR−/−</td>
<td>↓ 84%</td>
<td>Atherosclerosis decreased markedly at the inner curvature of the aorta (major area of disturbed flow in mice) with KO, but may actually be increased in other areas (626).</td>
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<tr>
<td>VEGFR2</td>
<td>VEGFR2 DNA vaccine in LDLR−/−</td>
<td>↓ 66%</td>
<td>Shown is the decrease in rate of progression for established lesions (vaccine at 22 wk). Also, 77% reduction in initiation of new lesions caused by carotid collars (734). Some report similar results (1412), while others report no change in lesions with anti-VEGFR2 (Flk1) treatment (1097).</td>
</tr>
<tr>
<td>Shc1</td>
<td>p66Shc−/− apoE−/−</td>
<td>↓ 60%</td>
<td>Mutation of only the N-terminal CH domain unique to p66Shc did not modify production of the p4B and p52 isoforms which have alternative, downstream initiation sites (1147). p66Shc mediates mitochondrial ROS production by many stimuli. 75.5% reduction in lesions was seen in mice without genetic hyperlipidemia fed a very high fat, high cholesterol diet (1283).</td>
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<tr>
<td>PLCβ2/3</td>
<td>PLCβ2/3−/− apoE−/−</td>
<td>↓ 51%</td>
<td>Strong effects on macrophage function seen as well (BMT suggests most of effect is macrophage-related) (1925).</td>
</tr>
<tr>
<td>ADAM15</td>
<td>ADAM15−/− apoE−/−</td>
<td>↓ 61%</td>
<td>ADAM15 associates with VE-cadherin. Its cytoplasmic tail binds Src and Yes which promote disruption of the adherens junction with increased endothelial permeability (1705).</td>
</tr>
<tr>
<td>FN</td>
<td>FN IIα−/− apoE−/−</td>
<td>↓ 53%</td>
<td>Adhesion of endothelial integrin α5β1 to FN (fibronectin) primes endothelial cells for an inflammatory response. The IIα domain also serves as a monocyte adhesion molecule and can be presented by the endothelial cell by apically expressed α5β1 integrin. Complete fibronectin KO is embryolethal. Increased fibronectin content of extracellular matrix is seen in areas of disturbed flow. IIα is a key domain of fibronectin (1751).</td>
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<tr>
<td>nmMLCK</td>
<td>nmMLCK−/− apoE−/−</td>
<td>↓ 31%</td>
<td>nmMLCK (nonmuscle isoform[s] of myosin light-chain kinase, encoded by MYLK1) is an effector of Rho/ROCK and other upstream kinases. nmMLCK phosphorylates myosin to activate actinomyosin contraction in endothelial cells (and leukocytes) and by a nonkinase mechanism activates Src with both effectors leading to increased endothelial permeability as well as monocyte diapedesis (1706).</td>
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<tr>
<td>BMP2</td>
<td>VSCM-specific BMP2 Tg in apoE−/−</td>
<td>NS</td>
<td>But greater intimal calcification seen due to induction of osteoblast-like cells (1276). Included here (though VSMC associated) because of BMP4.</td>
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<tr>
<td>BMP4</td>
<td>Fc-ALK3 intra-peritoneal administration into LDLR−/− mice</td>
<td>↓ 43%</td>
<td>BMP4 expression is increased by disturbed flow and induces NOX1 in endothelium (413). Inhibition of BMP2/4 signaling by a recombinant BMP ligand trap, Fc-ALK3, had a greater proportional effect than BMP2 over-expression, though this was in VSMC (above).</td>
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<tr>
<td>MGP</td>
<td>MGP human Tg in apoE−/−</td>
<td>↓ 37%</td>
<td>MGP (matrix Gla protein) is one of several endogenous inhibitors blocking BMP interaction with extracellular receptors (others include noggin and chordin). SMAD1/5/8 phosphorylation was decreased and much less calcification seen in these Western diet-fed mice (2029).</td>
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<td>MGP−/− apoE−/−</td>
<td>↓ 96%</td>
<td>MGP deficient, chow fed mice showed increased arterial calcification, but surprisingly, very little atherosclerosis with very few macrophages and nearly absent expression of ICAM-1, VCAM-1, or E-selectin (2029).</td>
</tr>
<tr>
<td>BMPER</td>
<td>BMPER+/− apoE−/−</td>
<td>↑ 31%</td>
<td>BMPER (BMP endothelial cell precursor-derived regulator) deficiency allowed increased BMP signaling and inflammation with markedly greater ICAM-1 and VCAM-1 expression (1418).</td>
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### Table 3.—Continued

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<tr>
<th>Gene</th>
<th>Model</th>
<th>Function, Comment</th>
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<tr>
<td>ANGPT2</td>
<td>ANGPT2 adenoviral-induced over-expression in apoE /−/</td>
<td>↓ 40% ANGPT2 (angiopoietin 2) is generally thought to promote inflammation by blocking</td>
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<td></td>
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<td>anti-inflammatory effects of ANGPT1 at the Tie2 receptor (88). However, NO</td>
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<td>production was stimulated and mediated the protective effect of ANGPT2 (20).</td>
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<td>Tie1</td>
<td>endothelial-specific Tie1 deletion [tamoxifen-responsive Cre-Lox] in</td>
<td>↓ 70% Tie1 (proline kinase with immunoglobulin and EGF factor homology domains 2) is</td>
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<td></td>
<td>apoE /−/</td>
<td>an angiopoietin [ANGPT] receptor that is upregulated in endothelium exposed to</td>
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<td></td>
<td></td>
<td>disturbed flow. Reduction in plaque shown is progression in aortic regions</td>
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<tr>
<td></td>
<td></td>
<td>exposed to disturbed flow. Little change seen at aortic root (1978). Tie1</td>
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<td></td>
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<td>activation by shear stress appeared to block the anti-inflammatory signaling of</td>
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<td></td>
<td>Tie2.</td>
</tr>
<tr>
<td>ERK5</td>
<td>endothelial-specific ERK5 deletion [tamoxifen-responsive Cre-Lox] in</td>
<td>↑ 130% In the same study, either elevated glucose plus low-dose hydrogen peroxide or</td>
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<td></td>
<td>LDLR /−/</td>
<td>higher hydrogen peroxide activated RSK1/2 binding to ERK5 with inactivating</td>
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<td></td>
<td></td>
<td>phosphorylation of ERK5 S496 by RSK1/2 (988).</td>
</tr>
<tr>
<td>SENP2</td>
<td>SENP2 /−/ LDLR /−/</td>
<td>↑ 97% SENP2 (sentrin/SUMO protease 2) desumoylates p53 and ERK5. Sumoylated p53</td>
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<tr>
<td></td>
<td></td>
<td>binds BcII and is pro-apoptotic whereas sumoylation of ERK5 is inhibitory. BMT</td>
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<td>experiments showed effect was due to vascular wall expression of SENP2. Much</td>
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<td>larger effect in areas of disturbed flow where sumoylation of p53 and ERK5 is</td>
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<td>increased (749).</td>
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<tr>
<td>KLF2</td>
<td>KLF2 /−/ apoE /−/</td>
<td>↑ 31% Effect of KLF2 placed here to emphasize endothelial effects even though</td>
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<td>hemizygous KLF2 deficiency was accompanied by a 39% increase in endothelial KLF4</td>
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<td>which may have largely compensated for any deficit in KLF2. No changes in eNOS or</td>
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<td>VCAM-1 expression were seen. There was an increase in FABP4 expression in</td>
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<td>macrophages that may have contributed to the increase in lesions (85). Hematopoietic</td>
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<td>cell-specific KLF2 deficiency in LDLR KO mice resulted in increased atherosclerosis</td>
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<td>with greater macrophage and neutrophil adherence to endothelium and activity (1050).</td>
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<tr>
<td>Arg2</td>
<td>endothelium-specific Tg human Arg2 in apoE /−/</td>
<td>↑ 79% Endothelial-cell-specific expression of human Arg2 (arginase II) decreased</td>
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<td>arginine availability for NO production by eNOS (1837). Arg2 is a mitochondrial</td>
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<td>enzyme.</td>
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<td>Arg2 /−/ apoE /−/</td>
<td>↓ 67% Arg2 /−/ macrophages were less responsive to proinflammatory stimuli and fewer</td>
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<td>entered plaque (1214). Arg2 /−/ macrophages in some way facilitates</td>
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<td>mitochondrial ROS production and is part of the M1 response.</td>
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<td>eNOS (NOS3)</td>
<td>eNOS /−/ apoE /−/</td>
<td>↑ 80% NO produced by eNOS mediates some of the protective effects related to</td>
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<td>as well as inhibition of cell adhesion. Deficiency also results in higher blood</td>
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<td>pressure (523). Transgenic over-expression of human eNOS lowered blood pressure (20</td>
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<td>mmHg) and decreased atherosclerosis by 40% (1855).</td>
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<td>nNOS (NOS1)</td>
<td>nNOS /−/ apoE /−/</td>
<td>↑ 31% Double KO mice lacked the α isoform of nNOS. Total en face plaque area was</td>
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<td>increased only in females at 24 wk (as shown). No effect on aortic sinus lesions</td>
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<td>seen (959). Interestingly, mice lacking eNOS, nNOS, and iNOS have greatly</td>
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<td>elevated LDL due to decreased hepatic LDLR expression and markedly</td>
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<td>accelerated atherosclerosis (2030). nNOS was recently shown to be a major</td>
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<td>source of hyperpolarizing, vasodilating hydrogen peroxide in response to shear</td>
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<td>stress while nNOS production of hydrogen peroxide was diminished with</td>
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<td></td>
<td>atherosclerosis (257).</td>
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<tr>
<td>GTPCH</td>
<td>GTPCH Tg apoE /−/</td>
<td>↓ 28% GTPCH (GTP cyclohydrolase I) is the rate limiting enzyme for BH4 synthesis.</td>
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<td>BH4 levels increase 3- to fourfold in endothelial cells of Tg mice (56). Lower levels</td>
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<td>of BH4 and increased eNOS uncoupling were seen in aging arteries (2013, 2027).</td>
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<tr>
<td>Cav1</td>
<td>Cav1 /−/ apoE /−/</td>
<td>↓ 33% Re-expression of Cav1 (caveolin) only in endothelium restored level of</td>
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<td></td>
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<td>atherosclerosis. Less LDL penetration and greater NO production was seen in</td>
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<td></td>
<td>Cav1 /−/ mice (522). Endothelial-specific overexpression increased</td>
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<td>atherosclerosis (521). Even greater reduction in atherosclerosis seen in a more</td>
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<td>recent study (477).</td>
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<td>NPR1</td>
<td>NPR1 /−/ apoE /−/</td>
<td>↑ 64% NPR1 (natriuretic peptide receptor 1) is a membrane-bound guanylate cyclase</td>
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<td>with actions presumably mediated through PKG). It serves as the receptor for both</td>
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<td>atrial and brain natriuretic peptides. NPR1 KO mice had higher blood pressure</td>
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<td>which may have accounted for increased plaque size. Plaques also had more</td>
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<td>VSMC, thicker fibrous caps, more calcium, but more cholesterol clefts (46). NPR1</td>
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<td>KO mice also have hypertension and cardiac hypertrophy.</td>
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<tr>
<th>Gene</th>
<th>Model</th>
<th>Function, Comment</th>
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</table>
| COX-1        | COX-1/−/− apoE/−/−                          | ↓ 74%  
Reduction in lesions was even greater in some measures (1167). COX-1 deletion virtually eliminated plasma TXA₂ (more than complete platelet COX-1 inhibition with aspirin), suggesting alternate sources of synthesis. Similarity to protection from TP KO (below) is striking. |
| COX-2        | Inducible Cre-lox COX-2/−/− in apoE/−/−     | ↑ 45%  
Inducible whole body COX-2 KO did not result in blood pressure or lipid changes. There was no excess atherosclerosis in mice with combined COX-2 and FLAP KO (2050). Macrophage-only KO by Cre-Lox in LDLR-null mice decreased atherosclerosis 25% at 6 mo but BMT of COX-2 KO marrow showed no effect in apoE KO mice (1284). |
| ERα (ESR1)   | ERα/−/− LDLR/−/−                           | ↑ 280%  
Shown is the increase in lesion area in ovariectomized ERα KO given the larger estrogen supplement (from subcutaneous pellets). BMT and Cre-Lox endothelial-specific KO studies showed the protective effect of the ERα was due endothelial effects (146). AMPK activation may be involved (2084). Estradiol in ovariectomized LDLR KO mice increased COX-2 and benefit was dependent on PGF₉ signaling through IP receptor (462). |
| CYP7B1       | CYP7B1/−/− apoE/−/−                         | ↑ 72%  
CYP7B1 converts 27-hydroxycholesterol (27HC) to 7,27-dihydroxycholesterol in the pathway to chenodeoxycholate synthesis. CYP7B1 deficiency results in accumulation of 27HC which inhibits both transcriptional and nonnuclear effects of ERα and ERβ (1833). Increased atherosclerosis also with 27HC administration (1832). |
| mPGES-1      | mPGES-1/−/− LDLR/−/−                       | ↓ 34%  
mPGES-1 (microsomal prostaglandin E synthase 1) is an inducible enzyme. It was primarily expressed in lesional macrophages and KO decreased PGE₂ synthesis mainly in macrophages with subsequent depletion of lesional macrophages. KO also resulted in increased PGI₂ synthesis by VSMC (1906). Data is placed here rather than table 6 because of the increased VSMC PGI₂ and to group this data with other prostaglandin-related genetic interventions. |
| IP           | IP/−/− apoE/−/−                            | ↑ 32%  
PGI₂ receptor (IP) is present on multiple cell types besides endothelial cells (925). Similar average increase seen in a study with LDLR KO mice (462). |
| TP           | TP/−/− apoE/−/−                            | ↓ 74%  
TXA₂ prostaglandin receptor (TP) placed in this table because of marked proinflammatory endothelial signaling (925). |
| DP1          | DP1/−/− apoE/−/−                           | ↑ 40%  
The PGI₃ receptor, DP1, is the target of the failed drug laropiprant, a DP1 antagonist. Later time points in apoE KO mice were nonsignificant (1888). Similarly, effect in LDLR KO mice was in females at 24 wk with lesser effects at later time and males (1654). DP1 KO in apoE deficient mice also increased aneurysm formation in the setting of prolonged ANG II infusion. Evidence was presented that the vasodilating DP1 receptor also had anti-platelet effects in humans (1654) |
| FP           | FP/−/− LDLR/−/−                            | ↓ 38%  
The F prostaglandin receptor (FP), also known as PTGFR (prostaglandin F receptor) binds PGF₃. Deletion of the receptor primarily affected renal sodium/water handling and thereby lowered blood pressure which appeared to explain the effect on atherosclerosis (2049). |
| fat-1        | fat-1 Tg in apoE/−/−                       | ↓ 38%  
fat-1 is a round worm gene that converts n-6 to n-3 fatty acids (1893). Markedly decreased inflammation was seen in lesions. |

**Table 3.—Continued**

**Endothelial protection with prolonged laminar flow–related to antioxidant defense**

<table>
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<tr>
<th>Gene</th>
<th>Function</th>
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| UCP1         | Aortic VSMC-specific overexpression of UCP1 in apoE/−/− | ↑ 350%  
Expression of UCP1 (uncoupling protein 1) in aortic smooth muscle unexpectedly led to increased ROS production (132). There was also a substantial increase in blood pressure and aldosterone which may have contributed to the increase in atherosclerosis. |
| UCP2         | UCP2/−/− C57BL/6J mice fed Paigen diet        | ↑ 321%  
UCP2 (uncoupling protein 2) is upregulated by oxidative stress or inflammation and dampens stimulated mitochondrial ROS production by an unknown mechanism. UCP2 KO mice fed a Paigen diet had increased VCAM-1 as well as other signs of endothelial activation (1240). UCP2/−/− BMT into LDLR/−/− resulted in modestly increased lesions with more macrophage apoptosis and signs of ROS-induced protein modification (157). UCP2 has since been shown to be crucial for macrophage efferocytosis as well (1380). |
| LIAS         | LIAS/−/− apoE/−/−                             | ↑ 43%  
Lipoic acid synthase (LIAS) produces lipoic acid in the mitochondria, a potent antioxidant which helps regenerate reduced glutathione. Homozygous lipoic acid synthase (LIAS) deficiency is embryo lethal. Only male LIAS−/− mice had an increase in lesion area (value given, females 5% increased, NS) and displayed increased TBARS and decreased erythrocyte GSH/GSSG which correlated strongly and inversely with lesion area in males (2037). |
Similar experimental design as for human catalase overexpression (above). Without weekly benzo(a)pyrene (in oil gavage, only catalase overexpression affected atherosclerosis. With benzo(a)pyrene, there was a marked increase in aortic F2-isoprostanes and reduction in atherosclerosis was significant for SOD1 overexpression as shown and additive with catalase (2022). VCAM-1 and ICAM-1 activation and leukocyte rolling (915).

MnSOD (also known as SOD2) converts mitochondrial superoxide to H2O2. Flow-mediated dilation was also reduced in these mice (2014). Homozygous knockout is embryonic lethal.

EC-SOD (extracellular superoxide dismutase) KO had only minor effects. Decrease in aortic surface area with lesions was only seen at 1 mo. By 3 and 5 mo there were no differences. No differences in LDL oxidation in aortic wall detected (1588).

HMOX1 (heme oxygenase 1) provides intracellular antioxidant defense in EC and VSMC (2035). Also important in macrophages to limit activation and scavenger receptor expression (1351). Induction of HMOX1 also appears to limit plaque vulnerability in rabbits (1035) and mice (301).

Over-expression of catalase reduced atherosclerosis. A twofold increase in catalase was significantly increased in 1 of the 3 black-6 mouse strains tested (1916).

Up to twofold increases in aortic plaque area were seen. BMT experiments showed similar contributions to atherosclerosis from either endothelial or leukocyte Prdx2 deficiency (1381).

In the apo E KO mouse Prdx6 KO did not affect atherosclerosis but atherosclerosis is embryonic lethal.

Aortic sinus at 20 wk in diabetic mice with GPx1 (glutathione peroxidase 1) KO (1016). Difference in nondiabetic mice was NS. Increased signs of oxidative damage seen.

Aortic sinus lesion area was strongly and directly proportional to aortic F2-isoprostanes. With benzo(a)pyrene, there was a marked increase in aortic F2-isoprostanes and reduction in atherosclerosis was significant for SOD1 overexpression as shown and additive with catalase (2022). VCAM-1 and ICAM-1 activation and leukocyte rolling (915).

GCLC is embryo lethal. Effect of macrophage-specific overexpression is given here (244).

Increased apoptosis (caspase 9 activity) of macrophages seen when exposed to acrolein accounted for by hematopoietic cells, presumably macrophages. Increased adhesion molecules (677).

Trx2 (thioredoxin 2) is the mitochondrial form in mice. VEGF promoter used for endothelial-specific over-expression (2070).

TTPA (tocopherol transfer protein, alpha, also α-TTP) is a vitamin E transfer protein involved in loading of VLDL with dietary vitamin E (delivered to the liver by chylomicrons and chylomicron remnants). Plasma and aortic tissue levels of vitamin E were reduced in TTPA KO mice and lesions correlated with F2-isoprostanes (1772).

GULO (gulonolactone oxidase) is found only as an inactive pseudogene in humans, primates, and guinea pigs. Increased atherosclerosis with dietary deficiency of both vitamin E and vitamin C in GULO KO, apo E-null mice was also shown in this study together with increased macrophage content. SVCT2 (sodium-vitamin C transporter 2) is a sodium-dependent transporter mediating vitamin C transport into many tissues including vascular cells. Prior studies in GULO KO, apo E-null mice were negative regardless of vitamin C intake, suggesting more severe tissue deficiency of vitamin C or combined vitamin C/vitamin E deficiency is required to promote atherosclerosis (83).
Another event occurring after onset of flow is exocytosis of Weibel-Palade bodies (WPB) with rapid cell-surface expression of presynthesized P-selectin (577). Weibel-Palade bodies are cigar-shaped vesicles unique to endothelial cells. In addition to P-selectin they contain von Willebrand factor (vWF), IL-8, pro-endothelin, angiopoietin 2 (ANGPT2), and other substances. Besides flow and histamine, agonists for WPB exocytosis include aldosterone (848), fibrinogen (1587), VEGF (through VEGFR2 not VEGFR1) (1996), chromogranin A (298), and peroxynitrite (632).

A mechanism for exocytosis has been proposed which provides a useful illustration of G protein signaling (1506). The Ca\(^{2+}\)-CaM complex binds to Ral-GDP dissociation stimulator (RalGDS, a GEF), inducing a conformation change that relieves autoinhibition and frees RalGDS from its cytoplasmic sequestration by β-arrestin. RalGDS moves to the membrane and binds active (GTP-bound) Ras, allowing RalGDS to exchange GDP for GTP on membrane-bound RalA (one of the Ras family of GTPases). Active RalA then mediates exocytosis of the WPB by coordinating the exocyst complex through enhancing activity of phospholipase D1 (PLD1). PLD1 cleaves choline from phosphatidylcholine to yield phosphatidic acid which promotes fusion of the vesicle and plasma membrane (1506).

3. Cytosolic phospholipase A\(_2\), platelet activating factor, lysophosphatidylcholine, and lysophosphatidic acid

Cytosolic PLA\(_2\) (cPLA\(_2\) or group IVA phospholipase A\(_2\)) is also depicted in Figure 7. Activation of cPLA\(_2\) depends on calcium-induced translocation to cellular membranes (especially the nuclear envelope and Golgi) and interaction with membrane PIP\(_2\) and/or ceramide-1-phosphate. Various kinases, such as PKCβ, CaMKII, or MAPK interacting kinase 1 (MNK1), can phosphorylate cPLA\(_2\) and thereby increase its activity (234). The products of cPLA\(_2\) are arachidonic acid (the precursor of the prostanooids) and lysophosphatidylcholine (LPC). LPC can give rise to lysophosphatic acid (LPA), and both LPC and LPA can bind to different GPCR and promote inflammatory signaling, although the identity of the LPC receptor or receptors remains controversial. LPC is also an important “find me” signal produced by apoptotic cells which summons phagocytes and promotes efferocytosis. Efferocytosis is normal endocytosis by apoptotic cells which summons phagocytes and promotes clearance of apoptotic cells by phagocytes. Surprisingly, knockout of cPLA\(_2\) led to a reduction in various inflammatory syndromes (916). This observation may or may not be relevant to atherosclerosis. Virtually all the prostaglandins can act as mediators or modulators of inflammation in various models. Even PGI\(_2\), typically thought of as anti-inflammatory, classically promotes acute inflammation by increasing endothelial permeability in small vessels (1286). Decreased VSMC proliferation after placement of a carotid cast was seen with cPLA\(_2\) deficiency, but the relationship with atherosclerosis is unclear (816). Recent data suggest that a calcium-insensitive PLA\(_2\) (iPLA2) may actually be the predominant isoform responsible for PGI\(_2\) synthesis in endothelial cells (1603), but its relationship to atherosclerosis is also unclear.

LPC may serve as a precursor for PAF synthesis. PAF was noted to be expressed rapidly on the surface of endothelial cells after various stimuli including histamine, thrombin, TNF-α, and leukotrienes (1075). Disturbed flow may also increase PAF expression or a similar product (Figure 7) (1152, 1425). Disturbed flow may actually increase expression of PAF receptors on endothelial cells (1337). Surprisingly, however, the relationship between atherosclerosis and expression of PAF or its receptor (which primarily affects neutrophil adhesion) has apparently not been explored.

LPC (as well as oxidized LDL or secreted PLA\(_2\)) also appears to induce expression of m-calpain in endothelial cells.
m-Calpain is a calcium-dependent, intracellular cysteine protease which affects endothelial permeability by cleaving VE-cadherin (also known as CDH5 for cadherin 5) and thereby disrupting the adherens junction. Inhibitors of m-calpain reduced atherosclerosis in LDLR- and ApoE-deficient mice (1222).

Another product of LPC is LPA, the simplest phospholipid. Endothelial cells produce LPA from LPC primarily by the action of the secreted enzyme ATX (autotaxon) (see FIGURE 7). LPA effects will be further discussed in relation to endothelial activation in response to hyperlipidemia.

4. The role of prostanoids in atherogenesis

Arachidonic acid, released from phospholipids by PLA₂ enzymes, can act as a substrate for enzymes representing three major “orthodox” eicosanoid pathways (391): 1) cyclooxygenase (COX) enzymes leading to prostaglandins, 2) lipoxygenases which yield hydroxyeicosatetraenoic acids (HETEs) and leukotrienes, and 3) certain cytochrome P-450 (CYP) enzymes, particularly of the CYP2C and CYP2J series, which yield epoxysitostrienoic acids (EETs).

In the prostaglandin pathway, COX-1 and COX-2 both yield PGH₂ (through the intermediate PGG₂). PGH₂ is then utilized by various prostaglandin synthases (or isomerases) such as thromboxane synthase (TXAS) and prostacyclin synthase (PGIS). In noninflamed endothelial cells, prostacyclin (PGI₂) is the exclusive detectable prostaglandin synthesized (236), whereas platelets make mostly thromboxane A₂ (TXA₂) but no PGI₂. PGI₂ is a strongly vasodilatory, anti-platelet, and often anti-inflammatory prostaglandin acting through the I prostanoid receptor (IP). TXA₂ promotes inflammation and platelet aggregation through thromboxane prostanoid receptor (TP) signaling (1286). The COX-1 isofrom is constitutively expressed in many cells, including platelets, macrophages, VSMC, and endothelial cells and is generally not affected by inflammation or flow. The COX-2 enzyme is only expressed after induction by inflammatory signals or in endothelial cells exposed to prolonged laminar flow. Consequent to this endothelial expression, COX-2 seems to be the predominant source of precursor PGH₂ for endothelial prostacyclin synthesis in humans without inflammatory disease (421, 662) and in mice (304). In human atherosclerotic lesions, both COX-1 and COX-2 are expressed with substantial COX-2 expression by activated macrophages (1562).

All prostaglandins transmit their signals by way of GPCR. Contractile prostanoid GPCR include TP, EP1, and FP. In humans there are two TP isoforms with both TPα and TPβ expressed on platelets while only TPβ is expressed on endothelial cells. In VSMC, contractile prostanoid receptors increase intracellular calcium and inhibit adenylate cyclase (AC) to promote smooth muscle contractions. TP in platelets is coupled primarily to Ga₁₂/₁₃ (leading to RhoA activation which mediates shape change) and Ga₉ (causing PKC activation and subsequent aggregation). IP, DP₁, EP₂, and EP₄ are relaxant receptors and activate AC (adenylate cyclase) through Ga₉ coupling (1286). IP in platelets also causes cAMP generation through Ga₉ coupling which inhibits platelet activation (614).

Because of drug-related controversies, a great deal of attention has focused on COX-1 and COX-2 even though their substrate and product are the same. Meta-analyses show a dose-dependent increase in risk of coronary events among patients taking COX-2-selective inhibitors, presumably because the greater inhibition of the endothelial-predominant COX-2 decreases protective PGI₂ while leaving platelet TXA₂ synthesis relatively unaffected (618, 1651, 1806). Recent advances in inducible knockouts show COX-2 deficiency usually does increase atherosclerosis while COX-1 deficiency resulted in a substantial reduction in lesions (see TABLE 3). In the meantime, relatively little attention has been paid to cell-specific signaling in relation to the individual prostaglandin receptors.

Effects of TXA₃, or more specifically, TP signaling are not simply restricted to platelets. Various endothelial stressors such as age, diabetes, and hypertension can lead to increased ROS, elevated intracellular calcium, and decreased NO production, all of which impair endothelial IP and increase TP expression and signaling. Furthermore, both of the endoperoxides (PGG₂ and PGH₂) and even PGI₂ (particularly when produced at high but physiologic levels) can act as agonists for TP, causing endothelial-dependent arterial contractions and possibly promoting inflammation (1866). Although PGIS predominates, endothelial cells can express all the prostaglandin synthases. Their levels of expression can vary by animal variety, with age, intracellular calcium, ROS, and NO exposure. Exposure of endothelial cells to high glucose caused increased peroxynitrate formation, nitration and inhibition of PGIS, and diversion of PGI₂ to inflammatory TP signaling with adhesion molecule expression (2123). Importantly, endothelial TXAS expression and TXA₂ synthesis increase with age and inflammation generally (albeit accompanied by increased PGI₂ synthesis) regardless of whether the precursors come from COX-1 or COX-2 (1866). In human embryonic kidney (HEK) cells and aortic VSMC, ROS production caused by TP ligation promotes intracellular TP maturation and a doubling of cell surface TP expression in a positive-feedback pathway (1966). In human vascular endothelial cells, TP ligation also causes changes in cell-surface fractalkine (CX3CL1) expression and release that promotes leukocyte adhesion (1798). Such changes also have been associated with progressing atherosclerosis with increased TXAS and TP seen in more advance lesions of LDLR KO mice (367).
Genetic mouse models have clarified a number of additional issues regarding prostaglandin synthesis, signaling, and atherosclerosis, although questions remain (662). Details of prostaglandin-related studies are summarized in Table 3. Whole body COX-1 KO did decrease atherosclerosis, but COX-1 deficiency in hematopoetic cells only actually increased atherosclerosis, probably because of a compensatory increase in macrophage COX-2 production. Consistent with this notion, transplant of bone marrow or fetal liver cells deficient for COX-2 mostly decreased atherosclerosis.

TP KO potently inhibited early atherosclerosis (by 74%) (925) and direct pharmacological inhibition of TP decreased atherosclerosis more effectively than aspirin in ApoE KO mice and rabbits (510, 614). More established atherosclerotic lesions may be less affected by TP inhibition (463). While TXA2 is the most active, other TP agonists include several other prostaglandins, PGH2, nonenzymatically generated 8-isoprostanes (whose production is increased in the presence of ROS), and HETE (510). Thus aspirin cannot eliminate TP ligands altogether despite effective blockade of TXA2 synthesis.

While COX-2 production of PGI2 and subsequent signaling is associated with prolonged laminar flow, it is presented here for continuity. Signaling through IP generally opposes TP effects and is protective. Nevertheless, the increase in atherosclerosis after IP KO in ApoE- or LDLR-deficient mice was relatively modest (~36%) (see Table 3) (462, 925). In LDLR-deficient mice, most of the atherogenic effect of IP KO was reported to be restricted to females (462). In LDLR-deficient female mice, estradiol administration greatly decreased atherosclerosis while this effect was nearly abrogated by deletion of IP (462).

Recent results suggest DP1 has modest antihypertensive, anti-platelet, and antiatherogenic effects (1654, 1688) which may provide insights into the failed HPS-THRIVE study, a major clinical trial testing the combination of niacin with the DP1 inhibitor laropiprant. The F prostanoioid receptor (FP) binds PGF2α, FP was not detected in aorta or atherosclerotic lesions, including macrophages. Nevertheless, deletion of the receptor affected renal sodium/water handling with a reduction in renin granules and mRNA in afferent arterioles and reduced renin release and plasma renin activity together with lowered blood pressure. This may explain the modest reduction in atherosclerosis seen with FP KO in LDLR-deficient mice (2049). KO of the platelet PGE2 receptor EP3 greatly decreased thrombosis after mechanical injury to plaques in ApoE-deficient mice (661). In platelets, EP3 is coupled to Goα, and acts to reduce cAMP and therefore increases platelet activation.

Arachidonic acid is metabolized by CYP2C and CYP2J subfamily enzymes to 5,6-EET or 11,12-EET and 8,9-EET or 14,15-EET, respectively. These EETs increase cAMP (like PGI2), but have additional strong anti-inflammatory effects, including NF-κB inhibition. They promote endothelial proliferation as well. The relevant intracellular signaling pathways are poorly understood. Some of their anti-inflammatory effects appear to be due to competitive inhibition of the TP receptor (121). Soluble epoxide hydrolase (sEH), encoded by Ephx2, converts EETs to much less active dihydro derivatives (410, 411). KO of sEH decreased injury-induced neointima formation in the femoral artery but not in the carotid artery of ApoE-deficient mice (1476). Inhibitors of sEH, some of which are undergoing clinical trials, appear to inhibit atherosclerosis in ApoE KO mice, particularly in the setting of infused ANG II (which downregulates CYP2C and CYP2J and increases sEH expression) (411, 1923).

Importantly, n-3 fatty acids substituting for arachidonate in most prostanoioid pathways yield less active chemotactic, proinflammatory, or vasoconstrictive products (391). The n-3 fatty acids also appear to play a particularly important role in synthesis of recently recognized inflammation-resolving lipid mediators named lipoxins, resolvins, and maresins. These novel prostanoioid pathways involve lipoygenases, cytochrome P-450 enzymes, and acetylated COX-2. Typically, one cell type synthesizes a precursor which is acted on by a different cell type to yield the final product (1593, 1666). Lipoxin A4 is derived from arachidontate, resolvin E1 from the n-3 eicosapentaenoic acid (EPA), resolvins D1 and D2 from docosahexaenoic acid (DHA), and maresins from DHA. These resolving lipids will be discussed further in section VII.C.5.

Most animals, including all mammals, lack the ability to convert n-9 or n-6 to n-3 fatty acids. Transgenic expression in mice of the roundworm fat-1 gene from Caenorhabditis elegans, capable of conversion of n-6 to n-3 fatty acids, increased n-3 levels five- to eightfold in ApoE-deficient mice and decreased atherosclerosis by nearly 50% (1893).
C. Endothelial Activation by Hemodynamic Factors: Cytoskeleton-Associated Signaling

1. Structural components of endothelial mechanotransduction

In addition to the GPCR and various channels discussed above, transduction of shear stress is mediated by tension transmitted from the glyocalyx on the luminal side through the actin microfibers, cytokeratin, and possibly microtubules, to the adherens junctions (formed largely by VE-cadherin) between adjacent endothelial cells and to the focal adhesion complex which includes integrins and syndecans which attach the cell to the subendothelial extracellular matrix. Some aspects of this signaling are shown in Figure 8.

Native endothelial cells, which are themselves only ~200 nm thick, display a glyocalyx composed of stiff heparan sulfate fibers extending typically 400 nm to as much as 4,000 nm into the bloodstream from syndecan-1 (280, 1845). The glyocalyx has important barrier functions and helps control adhesion of leukocytes and platelets but also acts as an exquisite fluid flow sensor. Extensive intracellular microfiber interconnections extend between the cytosolic domains of syndecan-1 to form the actin cortical web (1784, 1936). Hyaluronan is secreted and can associate with CD44 in caveolae but remains largely soluble, weaving in and out of the glyocalyx. Hyaluronan contributes to the fluid drag produced by the glyocalyx and helps trap chemokines secreted by the endothelial cells (1472). Nevertheless, inhibition of hyaluronan synthesis was found to increase atherosclerosis (1273). Heparinase treatment selectively removes heparan sulfate but not the syndecan-1 core protein and impairs NO release and many other signaling steps in response to shear stress; COX-2 and PGI2 responses are not affected, given their independent transduction reviewed above (281, 1369). The length of the glyocalyx inversely reflects the activation state of the endothelial cell, and its expression is impaired in typical static cell cultures (280). Indirectly measured glyocalyx length in humans was reduced by hypercholesterolemia and lengthened in response to cholesterol reduction with statins (964). Knockout of syndecan-1 resulted in increased atherosclerosis in ApoE-deficient mice (699).

In addition to cytokines, the glyocalyx also provides sites for binding the enzyme xanthine oxidoreductase (XOR). XOR is present in two forms: xanthine dehydrogenase (XDH) and xanthine oxidase (XO). Both XDH and XO catalyze the oxidation of hypoxanthine to xanthine and xanthine to uric acid by removal of two electrons at each step. The preferred electron acceptor for XDH is NAD (with conversion to NADH), while XO transfers electrons primarily to O2 with resulting superoxide formation. XDH is reversibly converted to XO by redox-sensitive formation of an internal Cys535-Cys992 disulfide bond (and possibly another internal disulfide bond), which alters the site where NADH binds near the cofactor FAD and opens access for molecular oxygen to the same site (1311). Thus a positive feedback or feed-forward pathway exists whereby superoxide, peroxynitrite, and other oxidizing electrophiles (1178) can promote still greater superoxide production by converting XDH to XO. Also, XO has greater affinity for glycoproteins than XDH. Thus an oxidizing microenvironment should promote increased glycoprotein-bound XO at the surface of endothelial cells.

Oscillatory (back and forth) shear stress promotes conversion of XDH to XO through activation of NADPH oxidase activity as shown in Figure 8, whereupon XO becomes the major source of superoxide (1177). Remarkably, both XO and compensatory antioxidant defenses are more abundant in large veins than arteries (1724). XO is generally thought to be the more prevalent form intracellularly, as in hepatocytes, but increased intracellular oxidative stress also converts XDH to XO (as seen in human ApoCIII overexpressing transgenic mice which are hypertriglyceridemic and have greater liver fat accumulation) (41). Intracellular XDH to XO conversion may also be controlled by calcium and phosphorylation (1178, 1364).

XO activity promotes endothelial dysfunction which is ameliorated by XOR inhibitors such as allopurinol (1364) and more especially, febuxostat (1125). XOR knockout studies are not feasible in mice as the pups are stunted and live only 4–6 wk, presumably due to renal failure (1334). Nevertheless, tungsten administration (a relatively specific inhibitor of XOR) to ApoE-deficient mice led to a Western type diet for 6 mo resulted in 83% reduction of aortic atherosclerosis (1568). More recently, allopurinol was also found to decrease atherosclerosis in ApoE null mice (by 45%) with further studies showing an important role for XOR in promoting foam cell formation (964). Thus XOR overexpression in macrophages increased lipoprotein and scavenger receptors including VLDLR, SR-A1, SR-B1, and SR-BII while decreasing expression of cholesterol transporters ABCA1 (ATP-binding cassette A1, the gene found deficient in Tangier disease) and ABCG1. Effects essentially opposite to these were seen with either XOR knockdown or allopurinol. Furthermore, allopurinol inhibited expression of IL-1β, IL-6, IL-12, TNF-α, VCAM-1, MCP-1, MMP2, and MMP9 after activation by incubation of macrophages with serum from Watanabe heritable hyperlipidemic rabbits (964). These findings suggest the participation of XOR in multiple steps of atherogenesis and the opportunity for further exploration of the use of inhibitors to prevent progression of atherosclerosis.

2. Endothelial mechanotransduction through the adherens junction: sensitizing integrins from the inside-out

Integrins are heterodimeric transmembrane proteins, at least 30 in all, consisting of 1 of 15 types of α chains and 1 of 7 types of β chains (1). Integrins often aggregate in
large arrays called focal adhesions which can also contain syndecans (particularly syndecan 4). Integrins are unique in their ability to make switchable (on/off) links with extracellular proteins (that is, the binding affinity of their extracellular domains can be controlled by signaling from within the cell, so-called “inside-out” signaling) while also being capable of transmitting signals resulting from extracellular ligand binding to the cell interior.
(“outside-in” signaling). The versatility of integrin signaling and control is perhaps best illustrated by white cell diapedesis. Chemokines first “turn on” the integrins by inside-out signaling (think of 2 folded arms moving to an outstretched position exposing grasping hands). Then, turned on integrins further activate the cell (outside-in signaling) as they alternatively clasp and release specific extracellular proteins. During this process, integrins and chemokines mediate intracellular signaling that guides the cell toward a chemotactic signal. As integrins have no intrinsic enzymatic activity, these feats are achieved by binding an array of adapter proteins to the relatively short intracellular tails of the integrin α and β subunits. Endothelial integrins are key to flow-mediated signaling.

In endothelial cells exposed to disturbed flow, the most proinflammatory signaling appears to emanate from integrins, including activation of JNK and NF-κB. But for this to occur, inside-out signaling must first activate integrins to their high-affinity state. Only activated integrins recognize and respond to protein ligands in the extracellular matrix (ECM). The adapter talin is considered to be the major mediator of integrin inside-out signaling. Binding of talin to the β integrin cytoplasmic tail disrupts electrostatic binding between integrin α and β cytoplasmic domains, thereby altering the external configuration (1605). Some prior reviews proposed the adapter vinculin as a mediator of inside-out signaling. However, vinculin does not bind integrins directly but instead binds activated talin. Both talin and vinculin bind actin microfibers, with mechanical tension on talin exposing more vinculin binding sites, increased talin integrin binding, and stabilization of the focal adhesion (653, 1389).

In white cells and platelets, inside-out signaling proceeds when chemokines bind their cognate GPCR or thrombin cleaves and thereby activates its receptor. These receptors then activate PI3K and PLC followed by IP3 and DAG production, calcium release, and PKC activation. Calcium and DAG (possibly assisted by PKC) activate a Ca2+DAG which acts as a GEF for Ras-related protein 1 (Rap1). Acting through the adapter RIAM (Rap1-interacting adapter molecule), Rap1 then activates talin by relieving autoinhibition (66). The role of this pathway in endothelial integrin activation with flow remains uncertain, but may occur through a different Rap1 adapter (261). Another major pathway for talin activation is through generation of the membrane phospholipid PIP2 or PIP3 which can also relieve talin autoinhibition.

A model of flow-induced endothelial integrin activation is presented in Figures 8 and 9. Abrupt onset of flow results in torsional forces transmitted through the cytoskeleton to mechanoreceptors in adherens junctions and to integrins and syndecans at the base of the cell (388, 1784). Laminar flow drags the luminal surface of the endothelial cells, causing a measurable leaning of cells in the direction of laminar flow. This tilt is not seen in areas of disturbed flow. Onset of flow abruptly changes the angle between cells, transmitting tension to the proteins that form the adherens junction (1188). Signals are then generated which require platelet and endothelial cell adhesion molecule 1 (PECAM-1), VE-cadherin, and vascular endothelial growth factor receptor 2 (VEGFR2, also called Flk-1) (1064, 1828). PECAM-1 appears to act as the true mechanical transducer and generates an array of signals.

Traction on PECAM-1 causes release of Gq/11 from the bradykinin receptor B2 within milliseconds. While described as an apical receptor in the prior section, an important portion, or perhaps most of the Gq/11 activation by flow occurs because of a physical association between the extracellular domains of PECAM-1 and the bradykinin B2 receptor (1358, 2033). In areas of disturbed flow, Gq/11 is generally found apart from the PECAM-1 complex, whereas in areas of rapid laminar flow it is found near PECAM-1. PECAM-1 KO decreased atherosclerosis substantially, but primarily in areas of disturbed flow (see Table 3). Indeed, the arteries of PECAM-1 KO mice lacked inflammatory markers in areas of disturbed flow (1828), and their endothelial cells showed no nuclear translocation or transactivation of NF-κB nor elaboration of adhesion molecules in response to disturbed flow (300). Interestingly, endothelial PECAM-1 deficiency results in increased atherosclerosis in areas normally protected from atherosclerosis by laminar flow (725). As discussed below, this may be due to the dual role for PECAM-1 in sensing flow generally with inflammatory signaling predominating at onset of flow or with flow reversal but anti-inflammatory signaling taking hold after persistent laminar flow. Furthermore, PECAM-1 on leukocytes was atherogenic for all areas of the vasculature, probably due to the role of PECAM-1 in facilitating homophilic interactions between endothelial cell and leukocytes during migration (725).

Bradykinin B2 receptor stimulation could theoretically activate IKK2 and NF-κB through the CBM pathway discussed in section IIIB5. However, a quantitatively more important pathway seems to involve inside-out integrin signaling, apparently through activation of PI3Ky. Besides generation of PIP3, PI3K enzymes can also act on PIP to generate PIP2 directly or after phosphatase action on PIP3 (1141, 1274). Identification of the PI3Ky isoform as the main source of flow-mediated PIP3 synthesis (625) clearly implicates a GPCR as the upstream signal, since other PI3K isoforms (that is type 1A isoforms, with type II and III forms less common or not relevant) are activated when their regulatory subunits bind phosphorytrosine residues. Activation of PI3Kγ would presumably result in PIP3 formation as well as PIP3, activation of talin and binding to the cytoplasmic domain of β integrins, completing inside-out integrin activation. Interestingly, blocking PI3Kγ activity abrogated...
flow-induced JNK activation, but not ERK activation, demonstrating at least two separate MAPK activation pathways emanating from PECAM-1, consistent with the model in FIGURES 8 AND 9.

Activation of tyrosine kinases through PECAM-1 mediates the activation of ERK with onset of flow. The mechanism is instructive and is relevant for subsequent flow-mediated signaling. Tyrosine phosphorylation of the β integrin cytoplasmic tail is also presumably required for talin binding and regulates competitive binding of inhibitors (66, 67), but the connection between PECAM-1 activation and integrin tyrosine phosphorylation is unclear.

Onset of flow causes PECAM-1 to activate the intrinsic tyrosine kinase activity of the VEGFR2 cytoplasmic domain without VEGF binding. Association of the Src family tyrosine kinase, Fyn (Fgr/Yes related novel protein) with PECAM-1 is required for this mechanotransduction (313), but further details are apparently unknown. In resting cells, both the tyrosine kinase COOH-terminal Src kinase (Csk) and inactive Src are bound in close proximity by phosphotyrosines on VE-cadherin. Csk maintains Src inactive by phosphorylating Src Tyr530 (in humans), causing Src to fold upon itself, masking its own catalytic site. (Thus Csk is a rather exceptional example of an inhibitory tyrosine kinase.) Activated VEGFR2 phosphorylates a specific tyrosine on VE-cadherin allowing recruitment of SHP2 (Src homology 2 domain-containing phosphatase 2) to VE-cadherin. SHP2 dephosphorylates the tyrosine which binds Csk and possibly also Src Tyr530. With the inhibitory activity of Csk relieved, Src further self-activates (684). Active Src then phosphorylates additional tyrosines on VEGFR2 and VE-cadherin, providing binding sites for the adapter protein Shc. Shc itself is also tyrosine phosphorylated by Src and possibly by Fyn. Phosphorylated Shc (the p46 and p52 isoforms) can then recruit the Grb2-Sos complex followed by Ras-Raf1-MEK1/2-ERK activation (1064, 1827, 1828).

As noted above, mechanical traction on PECAM-1 causes direct activation of the Src family kinase, Fyn found complexed with PECAM-1. Active Fyn phosphorylates a tyrosine in the PECAM-1 immunoreceptor tyrosine-based inhibitory motif (ITIM) domain, recruiting SHP2. The phosphatase activity of SHP2 on PECAM-1 leads to further downstream ERK activation (313, 1737), possibly by dephosphorylation and inhibition of Tie2 signaling (86, 565).

Additional aspects of VEGFR2 signaling are relevant. A particular VEGFR2 phosphotyrosine (Tyr1173 on mouse) can recruit either PI3Kα or PI3Kβ (class IA isoforms) or may serve as a docking site for PLCγ with release of DAG and IP3 followed by highly inflammatory PKC signaling. Interestingly, VEGFR2 phosphorylated on Tyr1173 can also transmigrate to the nucleus and induce its own transcription (1057). The E3 ubiquitin ligase Cbl (Casitas B-cell lymphoma) binds at nearby pY1052 on VEGFR2 and serves as an inflammation limiting factor by ubiquitinating PLCγ and targeting it for degradation (1450, 1533). Additionally, the VEGFR2 receptor can form a complex with integrins and its proximity may, in some way, facilitate integrin activation (1720).

In addition to VEGFR2 and VE-cadherin, Shc also binds tyrosine phosphorylated β integrin tails. Indeed, Shc activation seems to be a focal point of endothelial activation by disturbed flow (1720). Although p52Shc binds β integrins as soon as integrins are activated, Shc does not cause inside-out signaling (66). The PTB domain of Shc primarily binds integrin β3 phosphotyrosine 759 while the critical binding site for integrin activation by talin is phosphorylated Tyr 747 (66, 414).

3. VEGFR2 signaling also controls endothelial permeability

VE-cadherin homophilic interactions between endothelial cells largely control permeability of the endothelial layer. Disruption of the interdigitating VE-cadherin interactions allows both blood fluids and leukocytes to pass between endothelial cells, inciting inflammation and its four cardinal features: calor (heat), dolor (pain), rubor (redness), and tumor (swelling). Reduced levels of VE-cadherin at cell-cell junctions and increased endothelial permeability are seen in areas of disturbed flow and can be induced by back and forth, reciprocating flow in endothelial monolayers (1205). Besides the signaling reviewed above, VEGF binding to VEGFR2, as well as other inflammatory signals trigger Src and Src family kinases to tyrosine phosphorylate VE-cadherin, leading to disruption of catenin binding, activation of the Arf-GEF ARNO (Arf nucleoside binding site opener, also known as cytohesin 2), and activation of Arf6 (ADP ribosylation factor 6, a small G protein) which controls endocytosis of VE-cadherin and disruption of the adherens junction (see FIGURE 9). These junction disrupting activities are counteracted by signaling from the Robo4 receptor which binds extracellular Slit2 protein (863, 1068). Src activity alone is insufficient to disrupt the adherens junction (10). Catenins include α-catenin which binds F-actin, tying the adherens junction to the cytoskeleton, β-catenin which together with γ-catenin (junction plakoglobin) binds α-catenin, and δ-catenin (p120) which stabilizes VE-cadherin at the adherens junction (453). Free β-catenin, released by dissolution of the adherens junction, can translocate to the nucleus where it functions as a transcription co-activator in Wnt signaling (discussed below), supporting endothelial proliferation and migration in response to VEGF angiogenesis signaling. In tight junctions, active PKCβ, generated from VEGFR2 signaling, phosphorylates occludin, disrupting its binding with ZO-1 and promoting occludin ubiquitination and endocytic removal from the cell-cell junction (1253). ZO-1 redistributes away from the
cell membrane with disturbed flow while occludin expression actually decreases (1796).

Direct studies regarding effects on atherosclerosis of the above endothelial permeability regulating proteins are lacking. Recently however, ADAM15, another transmembrane protein that associates with VE-cadherin, was identified as having proatherogenic signaling effects that increased endothelial permeability (1705). ADAM15 expression is upregulated by cytokines and increased in areas of atherosclerotic plaques. KO of ADAM15 resulted in a 61% reduction in aortic sinus atherosclerosis area in ApoE-deficient mice. Surprisingly, the extracellular metalloproteinase or disintegrin domains did not mediate the atherogenic effects. Rather, the COOH-terminal cytoplasmic domain was found capable of activating Src and Yes (Yamaguchi Y73 virus, Esh avian sarcoma oncogene, a Src family tyrosine kinase) which mediate disruption of γ-catenin binding to VE-cadherin and dissolution of the adherens junction. These results should stimulate further examination of other proteins that control endothelial permeability.

4. Sensitized integrins signal from the outside-in through switchable scaffolds

Specific activated integrins recognize and bind different extracellular proteins and transmit various downstream signals back into the cell in “outside-in” signaling. When integrins bind their cognate ECM molecules, they aggregate (in phases) into large arrays called focal adhesions with numerous signaling and linker proteins (598). A very much simplified depiction is given in FIGURE 8 showing just one aspect of integrin signaling. In a recent review, focal adhesion proteins numbered at least 180 with 742 reported interactions (2055). The cytoplasmic tails of integrins directly bind at least 12 adapters (only 2 are shown in FIGURE 8) with layers of adapters, GEFs, GAPs, G proteins, tyrosine kinases, serine-threonine kinases, and assorted phosphatases binding thereafter. Some members of this “adhesome” act as signaling molecules while also dynamically linking...
the focal adhesion to actin microfibers, intermediate filaments, and microtubules.

An important theme illustrated by integrin signaling is the action of tyrosine kinases creating “switchable scaffolds” (1389). Scaffolds or adapter proteins recruited into these pathways are reversibly tyrosine phosphorylated, allowing subsequent signaling molecules to attach and generate downstream signals. Not shown in Figure 8 are the various inhibitors and scaffold phosphatases which turn off focal adhesion signaling.

The particular makeup of the ECM to which endothelial cells are attached has a dramatic effect on subsequent integrin signaling. Integrins \( \alpha_5\beta_1 \) and \( \alpha_5\beta_3 \) generate proinflammatory outside-in signaling when bound to fibronectin, vitronectin, or fibrinogen. Without these proteins in the ECM, integrin activation does not lead to inflammatory signaling (692, 1352). Normal ECM consists primarily of collagen IV and laminin. When endothelial cells were plated on collagen, the collagen-binding integrin \( \alpha_5\beta_1 \) was activated by onset of flow and led to increased production of \( \alpha_5\beta_1 \) (568). This contrasts with platelet activation mediated by collagen binding to integrin \( \alpha_{IIb}\beta_3 \). Deletion of the \( \alpha_5 \) integrin subunit did not affect atherosclerosis in ApoE KO mice (658), possibly due to overwhelming signaling through \( \alpha_5\beta_1 \) and \( \alpha_5\beta_3 \) integrins as fibronectin accumulated in atherosclerosis-prone areas with age. Conversely, stimulation of the \( \alpha_5\beta_1 \) and \( \alpha_5\beta_3 \) integrins by onset of flow over endothelial cells grown on fibronectin resulted in activation of PKC\( \alpha \), NF-\( \kappa B \), p38, and JNK which inhibited the anti-inflammatory signaling from integrin \( \alpha_5\beta_1 \) (568, 692, 1352, 1353, 1878). Resulting JNK expression primes endothelial cells for apoptosis in atherosclerosis-prone areas (285). Importantly, these inflammatory signals are largely mediated by G proteins Rac1 and Cdc42 and the tyrosine kinase PAK (1353). Also, PAK can tyrosine phosphorylate VE-cadherin and thereby increase endothelial permeability. In response to disturbed flow, JNK1 was recently shown to upregulate transcription of RelA through the action of the transcription factor ATF2, thus priming subsequent NF-\( \kappa B \) activation and showing important cross-talk between these inflammatory pathways. Both slow flow as well as slow combined with oscillatory flow led to increased RelA expression, but slow/oscillatory flow markedly increased nuclear localization of RelA together with nearly threefold greater expression of VCAM-1. High shear flow completely suppressed VCAM-1 expression (361).

The pathway for PAK activation may be outlined as follows. High-affinity binding of activated \( \alpha_5\beta_1 \) or \( \alpha_5\beta_3 \) integrins to fibronectin or vitronectin promotes binding of paxillin to the \( \alpha \) integrin cytoplasmic tail and FAK to talin and paxillin. FAK then self-phosphorylates, providing docking sites for proteins with SH2 and PTB domains including Src, PI3K, PLC\( \gamma \), Grb2, and p130CAS. Src and FAK further phosphorylate each other. Shear stress subtly stretches p130CAS and progressively exposes additional phosphorylation sites for Src (1544). Extensive p130CAS tyrosine phosphorylation is measurable within 300 ms (1265).

For maximum sensitivity to stretch stimulation, tensioning of actin stress fibers is required through RhoA-mediated tonic activation of myosin (552). The tyrosine kinase Ack (activated Cdc42-associated kinase) appears to have a role similar to FAK but is activated by Cdc42 and can activate RhoA through the RhoGEF Dbl (578, 1825).

Phosphorylated p130CAS recruits Crk (chicken tumor virus regulator kinase, erroneously named since it is actually an adapter with no kinase activity) which is also phosphorylated by Src. Phosphotyrosines on p130CAS and particularly Crk provide docking sites for numerous additional effectors and adapters. Among these are the DOCK180-ELMO1 complex (with DOCK180 acting as a GEF for Rac1 and ELMO1 inhibiting ubiquitination and degradation), Grb2-Sos, and Git (G protein-coupled receptor kinase-interacting protein). Git binds PAK-interacting exchange factor \( \beta \) (\( \beta\)PIX) and inactive PAK (1389), PAK ultimately self-activates after it is released by \( \beta\)PIX. First, however, \( \beta\)PIX mediates GTP exchange with Cdc42, then Cdc42-dependent release of PAK, followed by activation of Rac1. Thus \( \beta\)PIX serves as a sequential GEF for both Cdc42 and Rac1 (1767). \( \beta\)PIX and Rac1 also mediate generation of oxidative stress in response to arterial stretch from high blood pressure (1868). Once active, PAK can serve as a MAP4K for the JNK pathway and enhances NF-\( \kappa B \) signaling by directly promoting activity of NIK while enhancing the sensitivity of NIK to ROS (1353).

FAK is a remarkable signaling modulator, particularly in migrating cells. Its release from the focal adhesion leads to transactivation of the \( \alpha_6 \) subunit of NF-\( \kappa B \) in the nucleus (1414). FAK also seems to control several aspect of cell polarity. Rac1 and Cdc42 promote protrusion of the cell membrane by stimulating actin polymerization at the leading edge of migrating cells. These G proteins are also activated on the downstream side of endothelial cells at onset of flow. In contrast, RhoA causes contraction of the uropod at the trailing edge through stimulation of ROCK1 and ROCK2. Changes in paxillin phosphorylation over time may promote a transition of FAK conformation and binding affinities such that, in newly formed adhesions at the leading edge, FAK initially inhibits RhoA by activating RhoGAPs while activating Rac1 and Cdc42 as above. As the cell moves forward over the stationary focal adhesion, FAK switches to a RhoA activator by binding RhoGEFs, thereby promoting uropod retraction (399, 1800).
Syndecan-4 acts synergistically with integrins in focal adhesions as a strain- or flow-sensing molecule (123, 1235). Syndecan-4 attaches to fibronectin extracellularly while the cytoplasmic tail binds and is phosphorylated by Src and also binds paxillin and FAK. PKCα is recruited to syndecan-4 and is activated by a unique mechanism through PI3K rather than by DAG and calcium. Active PKCα can then phosphorylate and thereby inhibit a G-nucleotide disassociation inhibitor (GDI) for RhoA, allowing RhoA activation (442).

In FIGURE 8, activation of PKCζ by ROS is shown. Specifically, disturbed flow was found to increase peroxynitrite, which then promotes activation of PKCζ (by several possible direct or indirect means). Activated PKCζ can, in turn, bind to and activate the SUMO ligase PIAS4 (protein inhibitor of activated STAT4), thereby promoting sumoylation of nuclear p53, with translocation of sumoylated p53 to the cytoplasm where it binds Bcl-2 and Bcl-xL and promotes apoptosis (750, 1738). The sumoylation state of p53 may thus determine whether the net effects of p53 accumulation are cytoprotective or apoptotic. PIAS4 also promotes sumoylation of NEMO with subsequent greater activation of NF-κB (1103). Disturbed flow also promotes sumoylation and inhibition of ERK5, impairing its protective effects (749). The SUMO deconjugating enzyme SENP2 acts on p53 and ERK5 and has protective effect on atherosclerosis (749).

5. Fibronectin synthesis and other alterations of the ECM in areas of disturbed flow

The alteration of the ECM, with an increase in fibronectin, occurs early in atherosclerosis prone areas in both wild-type and ApoE-null mice as part of the cellular response to disrupted flow while deposition of fibrinogen seems to occur later. Increased ECM fibronectin and fibrinogen is found in human atherosclerotic lesions as well (693). Endothelial fibronectin production is controlled, at least in part, by Wnt signaling. Proliferation, migration, and survival of VSMC and other cells also can depend on Wnt signaling.

In canonical Wnt signaling, extracellular Wnt proteins (19 isoforms) bind to 1 of 11 mammalian Frizzled receptors with LDL receptor-like protein 5 (LRP5) or LRP6 acting as a coreceptor. In the absence of Wnt signaling, the intracellular β-catenin destruction complex actively phosphorylates free β-catenin, targeting it for proteosomal destruction, thus keeping free intracellular β-catenin levels very low. The β-catenin destruction complex is composed of scaffold proteins axin and adenomatous polyposis coli (APC), together with GSK-3β and CK1 casein kinase 1 (CK1, which “primes” GSK-3β) and PP2A. Note that β-catenin bound to the adherens junction apparatus is not targeted by the destruction complex. With a Wnt protein binding to Frizzled and LRP5/6, the intracellular domain of Frizzled binds Dishevelled which inhibits the phosphorylation activity of the β-catenin destruction complex while LRP5/6 binds axin, leading to axin degradation. The phosphorylation of β-catenin is thereby stopped while PP2A is freed to dephosphorylate β-catenin. The result is an increase in cytoplasmic and nuclear free β-catenin. Nuclear β-catenin displaces the corepressor TLE1 (transducin-like enhancer of split, or “Groucho” in Drosophila) from T-cell factor (TCF) and recruits XIAP to ubiquitinate TLE1 and target it for degradation (712). TCF and β-catenin can then promote transcription of multiple genes such as cyclin D1, myc, survivin, and IGF-I which facilitate proliferation, migration, and survival. Besides canonical Wnt signaling, non-canonical pathways can include G protein activation (much as a GPCR), and signaling through other coreceptors. Production of fibronectin and other ECM proteins by VSMC is also controlled by Wnt signaling (1208).

Disturbed flow promoted fibronectin production through increased β-catenin signaling in human umbilical vein endothelial cells (600). Fibroblasts also produce fibronectin in response to Wnt signaling (630). ECM fibronectin is found to be increased after injury and inflammation and may facilitate subsequent inflammatory responses in such areas. Fibronecctin knockout markedly decreased atherosclerosis (see TABLE 3). NF-κB and JNK induce endothelial fibronectin secretion through NF-κB and AP-1 binding elements in the promoter (505), suggesting multiple means for positive feedback as the artery ages or after various insults.

In areas of slow flow, there is proliferation of VSMC and inward remodeling. This appears to be mediated, at least in part, by HB-EGF signaling (see FIGURE 2) with activation of NADPH oxidase, NF-κB, and Erk1/2 (2072). Rather similar signaling and promotion of vascular VSMC migration result from ANG II signaling with transactivation of the EGFR receptor (465, 1187, 1245). While such signaling was thought particularly relevant to posttransplant arteriopathy, intimal VSMC could conceivably produce fibronectin that contributes to proinflammatory responses for endothelial cells and migrating white cells generally. Inflammatory cytokines such as IL-1 can also stimulate vascular VSMC to produce fibronectin (334).

A surprising source of fibronectin is the liver with deposition from plasma into atherosclerosis-prone areas of the arterial tree. Liver-specific deficiency of fibronectin substantially reduced atherosclerosis by ~50%, but also suppressed proliferation of VSMC and prevented formation of fibrous caps (1499).

6. Bone morphogenetic protein signaling associated with disturbed flow

The more than 20 bone morphogenetic protein (BMP) cytokines are members of the TGF superfamily and use SMADs for intracellular signaling. Initially, BMPs were shown capable of inducing ectopic bone formation, but further stud-
ies showed more extensive effects in regulation of development, cell proliferation, differentiation, and apoptosis in various cell types. BMPs are involved in promotion of angiogenesis and can promote osteoblast-like differentiation in dedifferentiated VSMC and other cells. BMP2 and BMP4 have been most extensively studied for their vascular effects. Extracellular BMP2/4 binds dimers of type II receptors, such as BMPRII, and instigates juxtaposition of type I receptor dimers such as activin receptor-like 1 (ALK2) followed by serine/threonine phosphorylation by the constitutively active type II receptor of the cytoplasmic tails of ALK2. Activated ALK2 then serine phosphorlylates SMAD1, -5, and -8, which form active transcription factors after dimerizing with SMAD4. BMP signaling is also characterized by the existence of a series of ligand traps or extracellular endogenous inhibitors which sequester BMPs and block interaction with the cognate receptors. These inhibitors have varying affinity for the different BMP proteins and include Noggin, follistatin, chordin, BMP endothelial cell precursor-derived regulator (BMPER, called cross-veinless 2 in Drosophila), Cerberus, gremlin, twisted gastrulation homolog 1 (TWSG1), and matrix Gla protein (MGP). Further controls are imposed by intracellular E3 ubiquitin ligases, SMAD ubiquitination regulatory factors (SMURFs), and phosphorylation by MAPKs. BMP signaling in endothelial cells clearly induces NOX1 (not NOX2) and promotes proinflammatory changes including expression of adhesion molecules, primarily attributable to the induction of ROS production (310, 855). BMP4 signaling can also induce HMOX1, thus providing some antioxidant balance (278).

BMP4 expression and release is greatly upregulated by oscillatory, disturbed flow, whereas BMP2 is not. As such, BMP4 may be an important means of maintaining oxidative stress and inflammatory activation of endothelium at atherosclerosis-prone sites. In contrast, BMP2 is produced in response to inflammation (such as in response to TNF-α) or high pressure (278). Both BMP2 and BMP4 were induced by JNK1 in mice at atheroprone sites (285). Also, incubation of human aortic endothelial cells in high glucose increased expression of both BMP4 and BMP2; their type I receptors ALK1, ALK2, ALK3, and ALK6; the type II BMP receptor BMPRII; and at least two extracellular inhibitors of BMP receptor binding, Noggin and MGP. Similar effects were seen in aortic endothelium of diabetic mice or rats accompanied by vascular calcification (185). BMP2 was also upregulated in endothelial cells incubated with oxLDL (413). Interestingly, prolonged laminar shear leads to SMAD6 and -7 phosphorylation which leads to greater SMAD6/7 inhibition of signaling by SMAD1, -5, and -8, thereby blocking the effects of BMP4 and BMP2 (855). In contrast, disturbed flow upregulates endothelial production of BMP ligand traps listed above (though not proportionately as much as BMP4), possibly as means to partly mitigate the effects of increased BMP4 production (278).

Recent manipulations of BMP signaling suggest a potentially promising means of mitigating atherosclerosis. MGP overexpression modestly decreased atherosclerosis (2029). Yet, surprisingly, MGP deficiency almost entirely blocked endothelial production of ICAM-1, VCAM-1, and E-selectin and almost entirely abrogated atherosclerosis (2029). These results suggest MGP may be acting in some role beyond a mere BMP ligand trap. In contrast, BMPER hemizygous deficiency clearly increased atherosclerosis (1416). Specific inhibition of BMP type I receptors (such as ALK2) using a small molecule (LDN-193189) reduced atherosclerosis 43% in LDLR-deficient mice with a similar reduction by use of recombinant ALK3-Fc which bound and inhibited BMP2 and BMP4 (413). Both interventions were well tolerated by these mice, and clinical trials may be warranted after further testing. Interestingly, VSMC-specific transgenic overproduction of BMP2 in ApoE-deficient mice did not increase atherosclerosis but greatly increased intimal calcification of arteries, demonstrating separability between atherogenic and calcific effects of BMPs (1276).

7. ER stress in endothelial cells

ER stress, also called the unfolded protein response, occurs in cells with increased or excessive protein synthesis demands and is aggravated by increased ROS. In general, ER stress is triggered by a relative deficiency of ER chaperones which direct normal folding of newly synthesized proteins within the ER. In times of lower demand, plentiful chaperones occupy binding sites of several ER membrane-bound receptors. These receptors transmit stress signals when not occupied as in times of increased utilization of chaperones. These signals can help compensate for the demands of increased protein synthesis or, if extreme, they can promote apoptosis. ER stress is discussed in more detail in section X.C.3.

Endothelial ER stress was found in areas of disturbed flow in both swine (329) and mice (506). BiP (immunoglobulin heavy chain-binding protein, also known as GRP78), the best studied ER chaperone and mediator of ER stress, could be induced by exposing cultured endothelial cells to disturbed flow with upregulation dependent on MAPK p38 and integrin α5β1 signaling (506). BiP upregulation is generally a protective, compensatory response to ER stress. X-box binding protein 1 (XBP1), another key regulator of the ER stress response, was also found to be upregulated in atherosclerosis-prone branch points in mice. Prolonged XBP1 signaling promoted apoptosis which was counterbalanced by overexpression of VE-cadherin (2060).

AMP-activated protein kinase (AMPK) is the master energy sensor and regulator of the cell. It is activated by an increase in the ratio of AMP/ATP as well as by hypoxia, ROS, peroxynitrite, hyperosmolality, and certain drugs including metformin and thiazolidinediones. Activation of AMPK has numerous effects with multiple protein targets, but among these is suppression of ER stress, possibly by decreas-
ing mitochondrial ROS, suppressing NOX, and activation of eNOS (435, 2084). Depletion or deletion of AMPK led to oxidative modification (at Cys674) and suppression of SERCA2 activity resulting in depletion of ER calcium, increased intracellular calcium, increased ER stress, and accelerated atherosclerosis in LDLR KO mice (435). This increased atherosclerosis could be attenuated by giving a small molecule chaperone, tauroursodeoxycholic acid, that inhibited ER stress.

Another consequence of ER stress is activation of heat shock proteins (HSPs) (771). HSPs act generally as protein chaperones to promote proper folding and other functions involving the active modification of protein configuration. Some HSPs act in the ER to promote protein folding for proper maturation of nascent proteins. Others may act in the cytosol or elsewhere. For example, consecutive, ATP-dependent modifications of steroid receptor configuration, first by HSP70, then by HSP90, are necessary to “prime” these receptors before they can bind the steroid hormone. HSP60 chaperones certain cytosolic proteins into the mitochondria. When cells are not under stress, HSPs are bound to heat shock transcription factor 1 (HSF1) in the cytoplasm. When misfolded proteins are present or the cell is stressed by a number of factors [including ROS (2094), oxidized lipids, or cytokines] HSPs bind to the misfolded protein (or are otherwise utilized in the cell), becoming separated from HSF1. HSF1 then forms a trimer, moves to the nucleus, and stimulates transcription of HSPs. In the case of HSP60, levels are clearly upregulated in endothelial cells in atherosclerosis-prone areas of slow flow, with increased expression on the luminal surface. Cell surface HSP60 has been proposed as a trigger for an autoimmune response which may be important in promoting atherosclerosis (1957).

HSPs are generally considered protective (1829). Indeed, an increase in HSP90, with its ability to stimulate eNOS, is considered one of the pleiotropic beneficial effects of statins (557). Unexpectedly however, atherosclerosis was decreased in ApoE-deficient mice given an inhibitor (which blocked the ATP binding site) of HSP90, possibly due to a decrease in inflammatory client proteins (including STAT3 and IKK) together with a compensatory increase in HSP70 (1109).

B. Overall effect of slow or disturbed flow on endothelial activation

The net effect of slow flow on endothelial signaling and activation was elegantly demonstrated by Shaik et al. (1600) (see Figure 10). Slow flow was seen to rapidly activate first FAK, then the terminal MAPK p38, then the downstream effector MSK1, and finally phosphorylation of the p65 unit of NF-κB (Figure 10A). Levels of these activated, phosphorylated signaling molecules diminished over time, suggesting negative feedback. At the same time, mRNA encoding the β3 integrin, and especially the p65 unit of NF-κB, were greatly increased. The 245-fold increase in p65 mRNA was reflected only partially in the much lesser increases of active p65 or nuclear NF-κB. Nevertheless, the large pool of p65 mRNA illustrates the heightened sensitivity of the pathway, poised for markedly enhanced activation in areas of slow flow. A marked increase in cytokine and chemokine production was also seen, both as tracked by cellular mRNA and as cytokines and chemokines released into the medium (see Figure 10C). Conversely, rapid flow resulted in a virtually complete suppression of these same cytokines and chemokines. Besides the changes in IL-8 and GROβ illustrated in Figure 10C, a whole host of cytokines and chemokines were found to be similarly regulated, resulting in a net proinflammatory signal induced by slow flow and an anti-inflammatory response to rapid flow (1600).

D. Transition to an Atherosclerosis-Resistant Endothelial Phenotype

Prolonged exposure of endothelial cells to rapid laminar flow results in a quiescent, atherosclerosis-resistant pheno-

![Figure 10](http://physrev.physiology.org)
type with frank unresponsiveness to even potent inflammatory cytokines such as TNF-α (1390, 2012) or IFN-γ (1812). The molecular mechanisms which mediate this transition have been the subject of intensive investigation (see FIGURE 11).

1. Endothelial quiescence through PKA and PKB activities

At the onset of flow, the adherens junction PECAM-1-VEGFR2-VE-cadherin complex was introduced as the proximate instigator of integrin inside-out signaling (with resultant JNK and NF-κB activation). With continued unidirectional flow, subsequent binding of Gab1 to the complex (through Grb2) may mark one of the earliest steps toward the transition to quiescence. Gab1 directs assembly and activation of a signaling complex that includes Src, PI3K, SHP2, PLCγ, and Akt/PKB (854, 982). This complex is also critical for protective ERK5 activation (see below) (1627) and is inhibited by cigarette smoke which inactivates VEGFR2 (460). Gab1 and subsequent SHP2 binding is critical for activation of PKA and activation of eNOS by flow (428). While its signaling is complex (1968), the net effect of Gab1 appeared protective as deletion of Gab1 led to a significant increase in atherosclerosis in ANGII-stressed, ApoE-deficient mice (765) (TABLE 1).

Phosphorylation by PKA is one of the major negative controls on PKA, thereby suppressing NF-κB activation (568). PKA is activated when integrins containing β1 bind to collagen and other normal basement membrane proteins (568). PKA inhibits BMP4 signaling and its subsequent activation of NOX1 (358). Upregulation of COX-2 by laminar flow with generation of PGI2 and autocrine signaling through the IP receptor activates PKA by generating cAMP. PKA, activated by cGMP (after NO activates soluble guanylate cyclase), can also phosphorylate PAK, disrupting PAK association with Nck1 (noncatalytic region of tyrosine kinase) and altering inflammatory signaling through MAP4K4 and other pathways dependent on Nck1 (556).

Akt/PKB inhibits forkhead box protein O3a (FoxO3a), FoxO1, as well as GSK-3β by serine/threonine phosphorylation as noted above under INSR signaling. Active GSK-3β phosphorylates cyclin D1, marking it for degradation and causing cell cycle arrest with increased susceptibility to apoptosis. Both these effects were blocked when GSK-3β was phosphorylated by PKB in response to VEGF2 signaling. Furthermore, PKB blocked endothelial susceptibility to apoptosis as well as induction of IL-1 and TNF-α in response to IL-1 administration, demonstrating an anti-inflammatory, prosurvival effect (1057).

Endothelial PKB activity stimulated by VEGF2 signaling is protective in yet another way. HDAC3 can shuttle in and out of the nucleus and serve as a scaffold for assembly of signaling complexes. With disturbed flow, expression of HDAC3 is increased in both cytoplasm and particularly in the nucleus (989). In contrast, laminar flow increases HDAC3 primarily in cytoplasm, since PKB phosphorylates HDAC3 thereby excluding it from the nucleus (2057). Nuclear HDAC3 can associate with nuclear factor erythroid 2-like related factor 2 (Nrf2), deacetylate Nrf2, and reduce Nrf2 DNA binding, thereby suppressing the transcription of antioxidant genes. During prolonged, rapid, unidirectional, pulsatile flow, deacetylation of Nrf2 by HDAC3 ceased with a marked reduction in HDAC3 expression generally, allowing full expression of antioxidant genes. Furthermore, activated PKB forms a complex with HDAC3 which promotes more prosurvival, anti-inflammatory signaling, such as repression of c-Jun and NF-κB. In some way, HDAC3 also increases the expression of the p85α subunit of PI3K (39). HDAC3 is critical for enhancing differentiation of endothelial progenitor cells and promoting survival of mature endothelial cells (39). Part of these effects are apparently mediated by deacetylation of p53 to increase its activity together with increased expression of the p53 target p21Cip (2059). The net effect of HDAC3 on atherosclerosis was tested by lentivirus knockdown of endothelial HDAC3 in aortic isografts transplanted to the carotid artery. There was marked endothelial cell loss, greatly increased formation of atherosclerosis-like lesions, and, in some cases, vessel rupture (2057).

PKB phosphorylation also excludes HDAC7 from the nucleus. In the nucleus, HDAC7 blocks transcription of protective MEF2C (989). In the cytoplasm, phosphorylated HDAC7 binds β-catenin and prevents its translocation to the nucleus (2100).

2. Endothelial quiescence through Tie2 signaling

Tie2 (tyrosine kinase with immunoglobulin and epidermal growth factor homology domain 2) is a receptor tyrosine kinase structurally similar to VEGFR2 (86). Tie2 signaling between adjacent cells, stimulated by angiopoietin 1 (ANGPT1), strongly supports endothelial quiescence and stability. Laminar shear stress promotes Tie2 phosphorylation and activation in direct proportion to the speed of flow (991). VEGF2 can further promote Tie2 activation by a complex mechanism involving cleavage of Tie1 (1635).

Remarkably, in isolated endothelial cells, ANGPT1 binding to Tie2 promotes activation and angiogenesis, whereas only in confluent cells (with intact cell-cell junctions) does ANGPT1 promote quiescence. The difference depends on NOTCH signaling and, apparently, the juxtaposition of vascular endothelial protein tyrosine phosphatase (VE-PTP), both of which only occur at intact cell-cell junctions. VE-PTP limits ERK1/2 activation by Tie2 signaling (1967). Active Tie2 does bind Grb2-Sos which could activate the ERK1/2 pathway (86). In confluent endothelial layers, ANGPT1 ligates and bridges Tie2 receptors on adjacent
cells. The phosphorylated Tie2 cytoplasmic domain binds and activates PI3K followed by PKB. Active PKB then inhibits GSK-3β, decreasing the phosphorylation and degradation of β-catenin. In endothelium, besides Wnt signaling, β-catenin can serve as a transcriptional coactivator to amplify NOTCH signaling by enhancing transcription of the NOTCH ligand DLL4 (delta-like 4). Transcription of Kruppel-like factor 2 (KLF2) and connexin 40 (Cx40) are increased with powerful antiapoptotic and anti-inflammatory effects (563, 2073). However, NOTCH signaling is remarkably diverse. In macrophages, NOTCH1 supports proinflammatory activation (69). Pharmacological inhibition of NOTCH signaling decreased atherosclerosis in ApoE-deficient mice apparently by decreased macrophage activation and reduced accumulation in lesions (69), while anti-DLL4 antibodies decreased atherosclerosis in LDLR KO mice (562).

Tie2 signaling through RhoA leads to downstream activation of mammalian diaphanous (mDia) which sequesters Src, thereby limiting endothelial permeability by blocking VEGFR2-mediated internalization of VE-cadherin. Active Src can also reduce expression of KLF2 (1907). Tie2 also inhibits NF-κB translocation by promoting A20 activity through A20-binding inhibitor of NF-κB (ABIN2) (86).

ANGPT1 is synthesized in VSMC and other perivascular cells. In contrast, ANGPT2, which mainly acts as an inhibitor to ANGPT1, is synthesized in endothelial cells. Indeed, without ANGPT2 release from Weibel-Palade bodies, endothelial cells would possibly remain unresponsive to most inflammatory signals. PKB is additionally anti-inflammatory by phosphorylating FoxO1, excluding it from the nucleus and thereby blocking the induction of ANGPT2 by FoxO1 (86). Nuclear FoxO1 also induces endothelial ICAM-1 and TNF-α expression in LDLR-deficient mice. Surprisingly, however, adenoviral overexpression of ANGPT2 in ApoE KO mice was atheroprotective, apparently through increased PKB and eNOS activity (20), while overexpression of ANGPT1 increased atherosclerosis in these mice, possibly by activation of leukocyte Tie2 (19). The interpretation of these results is complicated by potential differences in outcomes when angiopoietins are induced by adenoviral-induced overexpression versus more physiological levels. In humans, ANGPT2 correlated with other markers of inflammation and rheumatoid arthritis patients who subsequently developed cardiovascular disease had higher ANGPT2 levels at onset of their arthritis, but causality cannot be demonstrated in this study either (1948).

The Tie1 receptor seems to generally inhibit Tie2 effects, possibly through dimer formation with Tie2. Tie1 is upregulated in areas of disturbed flow (86). Endothelial-specific Tie1 deletion reduced atherosclerosis substantially in areas of disturbed flow (see Table 3) (1978).

### 3. Controlled oxidant signaling induces antioxidant defenses, leading to endothelial quiescence

While excess or prolonged superoxide production promotes cellular damage and apoptosis (1345, 1686), controlled production of superoxide and other ROS is critical for adequate induction of cellular antioxidant systems (FIGURE 11). Physiological ROS sources include NOX enzymes and mitochondria as reviewed above. Mild oxidant stress activates a host of antioxidant defense genes which share an antioxidant responsive element (ARE) in their promoters. The newer term electrophile response element (EpRE), rather than ARE, reflects the importance of electrophilic reactions in triggering this system (208). The transcription factor Nr2f is the major inducer of genes containing promoter ARE sequences. Importantly, nuclear Nr2f accumulation is equally upregulated by laminar and slow oscillatory (back and forth) flow, but subsequent signaling appears to be impaired in cells exposed to oscillatory flow, resulting in greatly diminished expression of ARE-containing genes.

Nr2f2 is bound in cytoplasm by Keap1 (Kelch-like erythroid-derived Cap-N-Collar-Homology-associated protein 1) in a complex that includes a Keap1 dimer together with the E3 ligase Cullin 3 (528, 1722). This complex directs ubiquitination of Nr2f2, targeting it for proteosomal degradation. Covalent modification of key cysteines on Keap1 by reactive electrophilic compounds disrupts the Keap1 complex with release of Nr2f. Relevant electrophiles include 4-HE and other oxidized membrane lipids, 15d-PGJ2, nitrated fatty acids, peroxynitrite, NO, and numerous naturally occurring substances such as cinnamaldehyde (see sect. III C1) (208). Recall that all these electrophiles also promote mitochondrial ROS production. Laminar flow induces COX-2 (1336) followed by increased 15d-PGJ2 availability. In turn, 15d-PGJ2 can covalently bind and inactivate Keap1, allowing nuclear accumulation of Nr2f2 (788, 1329). Remarkably, 15d-PGJ2 also covalently binds to and activates the PPARγ receptor as well as covalently modifying NF-κB to prevent its DNA binding (1517). Enhanced eNOS activity and NO availability leads to NO nitrosylation of Keap1 and Keap1 inactivation (709). Excessive inhibition of ROS formation or blocking at least modest accumulation of electrophilic byproducts results in reduced Nr2f availability and inadequate induction of cellular antioxidant defenses. Nevertheless, if ROS or reactive electrophile concentrations rise excessively, then proinflammatory and proapoptotic signaling is stimulated through ASK1 and other signaling as reviewed in section III C.

Nearly all cellular antioxidant defenses are induced by Nr2f2. These are only alluded to in FIGURE 11. Many of these antioxidant proteins are required for transition to a quiescent state. For example, glutaredoxin (Grx) was essential for flow-mediated upregulation of eNOS (1899), and HMOX1 induction decreased TNF-α release by endothelial...
A number of studies have shown increased inflammation and atherosclerosis after deletion of many antioxidant proteins (or protective effects with over-expression) (see Table 3). Surprisingly, however, whole body Nrf2 KO in mice fed a high-fat diet did not increase but rather decreased atherosclerosis, apparently because Nrf2 also supports expression of the CD36 scavenger receptors in macrophages with increased foam cell formation (1917). In a subsequent study with chow-fed mice, reduced atherosclerosis in Nrf2 KO was again seen, together with reduced CD36 in macrophages, fewer macrophages in plaques, but also lower plasma lipid levels and reduced hepatic lipid production. (106). Because the predominant effects on atherosclerosis for Nrf2 KO seem to relate to foam cell formation, results of these studies are included in Table 8.

Among the most important antioxidant defense mechanisms is the Trx-TrxR system. Trx has a dual role as an antioxidant enzyme (capable of regenerating reduced cysteine from protein cysteine disulfide bonds and reducing sulfenic acid) and as a redox-dependent inhibitor of ASK1 (289). Importantly, in oxidizing conditions, Trx is bound by thioredoxin-interacting protein (TXNIP) through disulfide linkage, allowing ASK1 to activate the JNK pathway. TXNIP is a scaffold protein belonging to the α-arrestin family. TXNIP can shuttle from the nucleus to either cytoplasm or mitochondria where it can inhibit different isoforms of Trx. TXNIP is markedly increased in endothelial cells in areas of disturbed flow (1918). In the nucleus during conditions of disturbed flow, TXNIP acts as a corepressor of KLF2 expression (possibly by recruiting transcription repressors like HDAC1/3 to the KLF2 promoter). Indeed, endothelial KO of TXNIP completely reversed the proinflammatory expression of ICAM-1 and VCAM-1 and inhibited leukocyte adhesion in areas of disturbed flow while overexpression abrogated the upregulation of KLF2 activity seen with laminar flow (1918). Furthermore, overexpression of TXNIP promotes apoptosis. In pancreatic beta cells, TXNIP was dramatically induced by high glucose and was a major mediator of glucotoxicity through a TXNIP-Trx2-ASK1 pathway (1546). TXNIP expression and nuclear localization are repressed by prolonged laminar flow, dependent on increased NO production (1571, 2012). Finally, RNS can directly nitrosylate Trx and increase its binding to and inactivation of ASK1 (130).

4. *eNOS upregulation by laminar flow is critical for endothelial quiescence*

Immediately after initiation of flow, there is a surge in the production of both NO and superoxide. For ~2 h after initiation of flow or with prolonged slow, oscillatory flow, the balance favors superoxide production with neutralization of the vasodilating effects of NO. After several hours of laminar flow, however, NO is favored through multiple mechanisms (see Figures 11 and 12) (451). Indeed, enhanced production of NO is one of the hallmarks of the atheroprotective state (105).

Enhanced NO production with prolonged laminar flow is mediated by a gradual increase in eNOS mRNA as well as multiple posttranslational modifications of the eNOS enzyme (such as binding to HSP90, calcineurin, and certain phosphorylations) all of which decrease the dependence on calcium and calcmodulin, and increase activity overall. Induction of eNOS is mediated by KLF2, ERK1/2 signaling, and surprisingly, NF-κB (101). Calcium-CaM seems to displace or disrupt inhibitory caveolin binding of eNOS (101). Activating phosphorylations of eNOS include Ser1177 (by Akt/PKB, AMPK, PKG, CaMKII), Ser615, Ser633 (by PKA), and Tyr81. Dephosphorylation of Thr495 also promotes activity (101). ERK1/2, whose activity is stimulated by even transient calcium elevations by way of CaMKII and PYK2, can also directly phosphorylate eNOS Ser633 to promote activity (1994). At low levels of intracellular calcium, plasma membrane calcium ATPase (PMCA) is bound in a complex to eNOS and calcineurin and can apparently phosphorylate eNOS Thr495 while also inhibiting calcineurin. As calcium rises, PMCA may be redirected to pump calcium out of the cell, leaving calcineurin free to dephosphorylate Thr495 on eNOS (780). Calcium-CaM bound to calcineurin can then activate NFAT as well. NFATc2 may promote eNOS transcription (2023).

Deacetylation of eNOS by SIRT1 further activates eNOS (1157). The target(s) of SIRT1 on eNOS are one or more specific lysines (possibly K494 and/or K504) in the calmod-
SIRT1 expression with flow promotes deacetylation of the p300 transcriptional domain of eNOS leading to enhanced calmodulin binding. The deacetylase HDAC3 reverses this acetylation (869).

NF-κB expression is generally low in atherosclerosis-resistant regions, while it is elevated in predisposed segments in mouse (696, 864) and pig (1391), and it increases with age in humans (432). Nitrosylation of NF-κB by NO directly inhibits IKK, stabilizes IκB, induces IκB mRNA, decreases NF-κB transport to the nucleus, and inhibits NF-κB binding to DNA (1477). Various electrophiles, particularly nitrofatty acids, can also inhibit NF-κB activation. Increased SIRT1 expression with flow promotes deacetylation of the p65 subunit of NF-κB, making it more susceptible to degradation. SIRT1 deacetylation of p300 results in sumoylation of NF-κB which may selectively decrease transcription of a subset of NF-κB target genes (197).

Complete suppression of NF-κB, however, does not seem to be advantageous, since NF-κB can also act to support an increase in eNOS synthesis and has anti-apoptotic effects (389). This Yin-Yang behavior is modified in the setting of prolonged laminar flow, where the proinflammatory actions of NF-κB are almost entirely abrogated or uncoupled (as seen by a marked suppression of VCAM-1, E-selectin, and IL-8 production in response to TNF-α), leaving the cytoprotective effects of NF-κB (including induction of MnSOD and eNOS) intact (1390). Recent insights into the mechanism of this transition suggests phosphorylation by PKB of the histone acetylator, p300/CBP (CREB binding protein, a homolog of p300) may lead to an alteration of the genes targeted by NF-κB, together with marked increase in eNOS transcription (294). In addition, increased expression of the transcription factor Erg was found in quiescent endothelium with downregulation by inflammation. Erg was found to bind promoter regions of multiple inflammatory genes and block NF-κB action at these sites (447).

While caveolin acts as an inhibitor of eNOS when bound to the enzyme, it has been suggested that caveolin may also function as a scaffold to assemble activating proteins, thus resulting in more effective activation of eNOS when the proper signals are transmitted (101). This may indeed be the case in some settings, but decreased atherosclerosis was seen after knockout of caveolin (522). An adaptor protein, striatin, can bind caveolin and appears to mediate binding of the estrogen receptor ERα (and probably other steroid receptors such as MR) to caveolae together with juxtaposition of a Go protein, possibly from the GPCR GPR30. Estradiol binding promotes assembly of this complex followed by activation of Src, PI3K, Akt/PKB, and phosphorylation of eNOS at Ser1177 (1190), one of the major nonnuclear endothelial signaling effects of estrogen. Administration of estradiol bound to a large nondegradable molecule which excluded it from the nucleus but allowed signaling through the striatin complex led to increased eNOS activity and reduced atherosclerosis in ApoE KO mice (271).

NO downregulates NADPH oxidase subunits after prolonged exposure to laminar flow. The decline in superoxide production with prolonged laminar flow, shown in Figure 12, was accompanied by an ~50% decrease in the expression of the activity-limiting gp91phox subunit of NADPH oxidase while eNOS protein expression was increased 3.5-fold. The reduction in gp91phox expression was shown to be NO-dependent, although the specific signal transduction pathway was not demonstrated (451). Laminar flow also inhibited activation of caspase 3 which in cells unexposed to flow...
cleaved PKCζ into a more active catalytic subunit termed CAT(ζ) which could then activate JNK (594).

Yet another NO-dependent pathway contributing to endothelial quiescence is the downregulation of AT1R by NO, together with upregulation of AT2R. Indeed, AT1R was found to be expressed exclusively in atherosclerosis-prone areas in mice while prolonged shear stress in human endothelial cells downregulated AT1R expression and abolished the ANG II-induced induction of VCAM-1 (1456). Phosphorylation of eNOS by both PKA and PKG supported these effects.

5. MAPK cross-talk in the transition to a quiescent state

Cross-talk between different MAPK pathway kinases appears to be another important mechanism whereby prolonged laminar flow induces a protective endothelial phenotype. ERK1/2 is increased by flow, and although it can support inflammation, its antiapoptotic effects (such as activating Bcl-2 and eNOS by phosphorylation) may be relevant for transition to a quiescent state. Nevertheless, activation of the ERK5 module is considered most important in this regard. As depicted in FIGURE 11, activation of MEK5 by flow inhibits activation of the pro-apoptotic, inflammatory JNK (130, 1029). MEK5 activates ERK5 which interferes with signaling downstream of JNK (130). Of primary importance is the upregulation of KLF2 and KLF4 by ERK5 signaling through phosphorylation and activation of the transcription factor MEF2C (MADS box transcription enhancer factor 2C) (330, 1332, 1881). Laminar flow further supports MEF2C action by calcium/CaM-dependent export of inhibitory HDAC5 from the nucleus (1912). Inhibitory HDAC7 can be excluded from the nucleus by PKB phosphorylation (989). ERK5 also phosphorylates and activates eNOS, inactivates the proapoptotic factor Bad, and displaces the corepressor SMRT (silencing mediator for retinoid and thyroid hormone receptors), thereby allowing transcription mediated by PPARγ (154). PKCζ, which is activated by TNF-α, binds and inhibits ERK5 by phosphorylation (1306). ROS and advanced glycosylation end-products (AGE) appear to promote ERK5 sumoylation and inactivation, a potentially important mechanism for diabetes-related endothelial dysfunction (1977). High blood sugar and mild hydrogen peroxide exposure together or high hydrogen peroxide alone increased direct physical binding of RSK1/2 to ERK5. RSK1/2 then phosphorylated ERK5 on S496 and inhibited ERK5 transcriptional activity followed by increased VCAM-1 and E-selection expression while eNOS was decreased. Inhibition of RSK1/2 activity with the specific inhibitor fluoromethyl ketone-methoxyethylamine resulted in reduced atherosclerosis in ANG II-treated ApoE-deficient mice (986). In contrast, endothelial-specific, tamoxifen-induced conditional KO of ERK5 led to a 130% increase in atherosclerosis in LDLR−/− mice (986).

Despite the importance of the MEK5-ERK5 pathway, the mechanism of activation by laminar flow remains unclear but includes activation of AMPK (2046). A proposed pathway includes increased NO which binds and activates soluble guanylate cyclase (sGC) with production of cGMP and presumed activation of PKG, phosphorylation and activation of an L-type voltage-operated calcium channel, increased intracellular calcium and activation of CaMKII which can then phosphorylate and activate AMPK (2074).

6. KLF2/4, atheroprotective molecular switches turned on by laminar flow

Numerous studies have pointed to the induction of the transcription factor KLF2 (with KLF4 more recently added) as a critical step in the conversion to and maintenance of an athero-protective state (1106, 1332, 1881), even in microvessels (330). Indeed, KLF2 (and possibly KLF4) and Nrf2 are thought to act together to control as many as 70% of the genes upregulated by laminar flow (1291). In human, mouse, rat (1907), and swine (500) arteries, KLF2 (and KLF4 when examined) was found to be expressed by endothelial cells in areas of normal laminar flow but nearly absent in areas of slow or disturbed flow. KLF2 is also important in embryonic vascular and hematopoietic development. The molecular effects of KLF2/4 are protean and include the following: inhibition of ATF2 (activating transcription factor 2), a potential component of pro-inflammatory AP-1 (activator protein-1) (529); induction of inhibitory SMAD7 which blocks TGF-β signaling (177); enhanced transcription of eNOS and the enzyme dimethylarginine dimethylaminoiodohydrolase which degrades the eNOS inhibitor ADMA (asymmetrical dimethyl arginine); increased transcription of protective thrombomodulin (TM) while inhibiting transcription of tissue factor (TF), vWF, plasminogen activator inhibitor 1 (PAI-1), and the thrombin receptor protease activated receptor 1 (PAR1) (1049); increased expression of CD59 which blocks effects of complement activation on endothelial cells (911); inhibition of inflammatory effects of endothelin-1 (ET-1), MCP-1, IL-1β, TNF-α, and other inflammatory cytokines through inhibition of NF-κB; and a number of other major effects (1291). In one study, KLF2 decreased by 80–90% the expression of the following genes after exposing cells to IL-1β: IL-1α, IL-1β, IL-6, IL-8, IL-15, MCP-1, E-selectin, TNF-α, CXCL10, CXCL11, IFN-γ, COX-2, and CCL5 (1388). Inhibition of NF-κB by KLF2 occurs, at least in part, by KLF2 competing for CREB-binding protein (CBP) which is required for full NF-κB activity. Conversely, the p65 subunit of NF-κB may inhibit transcription of KLF2 (but not KLF4). This suggests that the balance of NF-κB and KLF2 may control the overall inflammatory status of endothelial cells (1291). Interestingly, statin drugs are potent inducers of KLF2 and KLF4, possibly by way of PKB activation (1332, 1388).

KLF2 upregulation by flow in endothelial cells resulted in increased expression of miR-143 by 17-fold and miR-145 by 12-fold, microRNAs known to be involved in control of
Several aspects of the regulation of KLF2 expression are shown in Figure 11. As noted above, a major inducer of KLF2 (1291) and KLF4 (330) is ERK5 which phosphorylates the transcription factors MEF2A (MADS box transcription enhancer factor 2, polypeptide A) and MEF2C among numerous other targets. HDACs generally act as corepressors by removing acetyl groups from histones, thus promoting a compact configuration of chromatin, preventing transcription. HDACs also remove acetyl groups from transcription factors and other proteins which may deactivate the target or may allowing tagging the same site with a phosphate to mark the protein for ubiquitination and degradation. HDAC5 is a co-repressor of MEF2A-mediated transcription of KLF2. Endothelial HDAC5 is phosphorylated and transported out of the nucleus by a calcium/CaM-dependent mechanism which is stimulated by laminar shear stress (1912). Cilia on endothelial cells can generate calcium signals under flow and recently were shown to be important for flow-induced upregulation of KLF2/4 (464). An additional mechanism to upregulate KLF2 activity involves activation of PCAF (p300/CBP associated factor) by a PI3K-dependent but PKB-independent mechanism (799). Tie2 signaling is thought to contribute to PCAF activation through activation of PI3K. Activated PCAF acts together with CBP/p300 to acetylate histones and greatly increase transcription of KLF2 and KLF2-regulated genes.

Factors that inhibit KLF2 expression (but not KLF4 which tends to be increased by inflammatory signals) include multiple pro-inflammatory cytokines which mediate translocation of active NF-κB to the nucleus, as noted above (1291). In addition, active Src decreases expression of KLF2 and may be another means whereby KLF2 is downregulated by disturbed flow (1907). Recently, miR-92a was shown to inhibit KLF2 and KLF4 translation and was found to be downregulated by laminar flow (500, 1988).

Many of the observations regarding KLF2/4 signaling have thus far been made in vitro or in artificial systems. Because KLF2 KO is embryonic lethal, one study examined KLF2 hemizygous KO in ApoE-deficient mice. Surprisingly, there was little effect on KLF2 target genes in endothelium, presumably because of an apparently compensatory upregulation of KLF4. However, there was a mild increase in atherosclerosis associated with increased macrophage lipid accumulation (85). More recently, hematopoietic deficiency of KLF2 increased atherosclerosis more substantially due to increased neutrophil and macrophage adherence and entry into lesions and increase neutrophil activity together with accelerated apoptosis (1050). Greater understanding of the role of KLF2 and KLF4 on endothelial-mediated pathways in atherosclerosis await additional studies such as conditional KO in endothelium.

E. Hydrogen Sulfide Signaling

Hydrogen sulfide (H₂S) is now recognized as a third gaseous transmitter/signaling molecule (after NO and carbon monoxide) (1264). The gas is produced from cysteine by the enzymes cystathionine β-synthase and cystathionase. Although H₂S is a lethal toxin at higher exposure levels (by inhibition of cytochrome c and cellular respiration), accumulating evidence suggests an anti-inflammatory, antioxidant signaling role at lower, physiological concentrations. Like NO, exposure to high H₂S (100–250 μM) causes oxidative stress and impairs survival in both endothelial cells and VSMC. Conversely, H₂S at low concentrations (30 μM for example) protects cells from various stressors such as hypophosphatidylserine (LPS), ischemia-reperfusion, H₂O₂, and high homocysteine, while reducing Nfkb signaling, cell ROS production, increasing antioxidant enzyme expression, and markedly improving cell viability (1028, 1098). Endothelial release of H₂S can parallel and complement NO.

H₂S promotes Nrf2 localization to the nucleus, inducing expression of multiple cellular antioxidant systems (1028). Providing exogenous H₂S in various in vitro studies reduced multiple aspects of early atherogenesis including endothelial inflammatory signaling and elaboration of adhesion molecules, monocyte adhesion and activation, as well as foam cell formation (1098). The primary mechanism of H₂S signaling seems to be protein cysteine sulfhydration (1263, 1264), although it is not clear that sulfhydration explains all its effects. For example, H₂S causes vasodilation by opening VSMC KATP channels (it is therefore an endothelial-derived hyperpolarizing factor). Phosphodiesterase inhibition with persistance of cGMP and cAMP signaling may be an additional important mechanism (1263, 1909). Notably, the type 3 phosphodiesterase inhibitor cilostazol was recently reported to cause carotid intima-media thickness (CIMT) regression among type 2 DM patients (888). H₂S reacts with intracellular S-nitrosothiols to form the smallest S-nitrosothiol (HSNO), and a role for hydrogen sulfide in controlling the intracellular S-nitrosothiol pool has been suggested.

Among the most exciting byproducts of H₂S signaling research have been major insights into hyperhomocysteinemia as a coronary risk factor. Despite numerous observational studies reporting increased CAD risk associated with elevated Hcy (homocysteine) (802, 1368, 1782), vitamin interventions have been universally negative despite effective Hcy lowering (333). A likely explanation for the apparent discrepancy is that elevated Hcy is not the culprit but rather a marker for defective H₂S synthesis (118). Two of the major enzymes of the transsulfuration pathway not only metabolize Hcy but are major sources of H₂S. Deficiency of
either cystathionine β-synthase (CBS) or cystathionase or cystathionine γ-lyase (CTH) markedly impairs cellular H$_2$S production. CTH KO caused hypertension mediated by impaired H$_2$S production (2020).

In one recent study in ApoE KO mice (2067), high early mortality seen in prior studies of CBS$^{-/-}$ mice was avoided by utilizing a zinc-inducible human CBS transgene in otherwise CBS-deficient, apoE$^{-/-}$ mice. During pregnancy and weaning, zinc was added to the drinking water, the transgene was activated, and Hcy levels were only slightly elevated, preventing early mortality. After weaning, zinc was removed from drinking water and the mice developed severe hyperhomocysteinemia and accelerated atherosclerosis, confirming prior observations in conventional CBS$^{-/-}$ apoE$^{-/-}$ mice (1896). Furthermore, lesion size and elevations in TNF-α, MCP-1, and Ly-6C$^{hi}$ monocytes/macrophages were all proportional to Hcy levels. However, insights from other studies suggest that impaired H$_2$S was likely the underlying causal factor. Thus in vitro studies showed that elevated plasma Hcy inhibited CBS and impaired H$_2$S production while H$_2$S supplementation completely reversed inflammation caused by hyperhomocysteinemia (1586). Chemical inhibition of CTH in ApoE KO mice caused a marked increase in atherosclerosis. This excess lesion formation was decreased (by 38%) by sodium hydrosulfide supplementation (1921). In another study, sulfur dioxide supplementation markedly decreased lesion development in atherogenic diet-fed rats (1037). Thus much of the prior work on Hcy in animal models of atherosclerosis needs to be reconsidered in light of newer understanding of H$_2$S signaling.

A number of human intervention trials are underway utilizing various H$_2$S donors (1028, 1098). Interestingly, naturally occurring sulfur compounds in garlic yield biologically meaningful levels H$_2$S by interacting with GSH in red blood cells (124). These insights may help explain positive cardiovascular outcomes of several smaller, randomized controlled trials that utilized garlic or garlic extracts. Results were largely independent of any effects on standard risk factors (222, 223, 247, 939).

F. Genomic Studies of Flow-Mediated Change in Endothelial Phenotype

Several groups have examined RNA expression profiles of 10,000 or more genes simultaneously in endothelial cells exposed to the atherosclerosis-prone versus protective flow patterns (212, 324, 375, 403, 528, 529, 590, 1391, 2045). While results generally confirmed the findings of candidate gene and molecular studies, there were many differences between the studies in specific genes identified as up- or down-regulated, possibly owing to differences in technical approach. Genes known to be associated with inflammation and susceptibility to atherosclerosis tended to be increased in cells exposed to the slow-flow, oscillatory pattern (disturbed flow), whereas they were suppressed in the cells exposed to rapid, unidirectional pulsatile flow. For example, after exposure to disturbed flow there was increased mRNA for IL-1α, IL-1α receptor, IL-6, IL-8, Cx43, VCAM-1 (with enhanced response to IL-1 stimulation), E-selectin, MCP-1, fibronectin, vWF, NADPH oxidase, MMP-1, and MMP-10 while some protective genes such as eNOS, IL-10, and chemokine CXCR4 were downregulated. A host of antioxidant genes were upregulated by the concerted actions of laminar flow, and these changes were associated with greater expression of KLF2 and Nrf2 (528, 529). Besides confirming effects on genes with known links to inflammation, these studies also provided many new candidate genes to be considered for their potential roles in the already complex molecular biology of endothelial responses to hemodynamic factors and subsequent atherosclerosis susceptibility.

In one of the studies, endothelial cells were harvested from atherosclerosis-prone and atherosclerosis protected areas of aorta from normal pigs (387, 1391). This in vivo approach suggested that while pro-inflammatory genes were frequently increased in the atherosclerosis-prone areas, several compensatory genes were also upregulated, including several antioxidant genes such as glutathione peroxidase (Gpx). Arteries from these normocholesterolemic pigs showed little evidence of frank inflammation, even at predisposed sites. Nuclear NF-κB levels were not elevated nor were several key adhesion receptors overexpressed on cell surfaces. Similarly, in chow-fed mice there was no excess nuclear NF-κB seen in endothelial cells taken from areas of disturbed flow. However, substantially greater levels of cytoplasmic NF-κB bound by IκB were evident. There was much greater activation of NF-κB and its inducible gene products in these same hemodynamically prone areas after the mice were treated with lipopolysaccharide or after feeding LDLR-deficient mice a high-fat diet to induce hyperlipidemia (696). Thus frank inflammation may not be present in areas of disturbed flow without additional initiating factors. Instead, disturbed flow seems to prime the endothelium for an enhanced response to activating or inflammatory stimuli.

V. INITIATION OF ATHEROSCLEROSIS: ENDOTHELIAL ACTIVATION BY DYSLIPIDEMIA

Striking electron micrographs reveal monocytes adhering to endothelium in atherosclerosis-prone areas just days after inducing severe hypercholesterolemia by feeding animals high saturated fat diets (with butter, lard, or coconut oil) enriched with added cholesterol (492, 493, 1511, 1577). Such hyperlipidemia clearly provides one or more signals for activation of already “primed” endothelial cells found in atherosclerosis-prone areas (26, 365, 696, 1023). Furthermore, lowering serum cholesterol, even if by only dietary means, rapidly decreases endothelial activation and
inflammation (1023). As early as 2 h after injection of human LDL into rabbits, grapelike clusters of aggregated LDL could be seen enmeshed in focal areas of the subendothelial matrix (1305). VCAM-1 and MCP-1 were expressed by endothelial cells within 3 wk of starting a high-cholesterol, coconut oil-based diet in rabbits (1023). The events occurring during these weeks have been the object of intense investigation.

Not only do lipoproteins preferentially accumulate in atherosclerosis-prone areas, but hyperlipidemia itself clearly increases the permeability of the endothelium and total area of susceptibility, suggesting direct activation (1577, 1578). Mechanisms for increased permeability include alterations in adherens junction function with increased paracellular permeability, greater susceptibility to apoptosis with increased cell turnover resulting in physical gaps in the endothelium, diminished glycocalyx height, and other factors (1843). Activation of endothelial RhoA by multiple mechanisms is known to assist in opening endothelial paracellular junctions (1172). Even modest hyperlipidemia induced by a high-cholesterol diet in pigs (increasing total serum cholesterol from 74 to 187 mg/dl) tripled endothelial labeled tythidine uptake, an index of endothelial cell turnover (531). Several possible mechanisms for endothelial activation by hyperlipidemia are presented below, and relevant genes are presented in Table 4.

A. LDL Oxidation: An Uncertain Role

Despite continuing skepticism (1685, 1686), much evidence supports a role for LDL oxidation in endothelial activation (1288, 1303). In human fetuses, native LDL appeared to accumulate in the intima before macrophages were found. LDL bearing oxidation-related epitopes seemed to accumulate only after native LDL were present, and such oxidized LDL were most frequently seen together with macrophages, although they could also be observed in the absence of macrophages (1282). In classic in vitro studies, severely oxidized LDL (oxLDL) (produced usually in vitro with non-physiological exposure to copper or iron ions) was shown to stimulate adhesion of both macrophages and T-cells to endothelial cells, promote diapedesis into the subendothelium, and lead to the arrest of egress (1288, 1445).

Despite the potential to initiate endothelial activation, oxLDL are thought to be found primarily in more advanced lesions, primarily in association with macrophages. Furthermore, oxidized LDL in human plaque appears to be formed primarily by exposure to myeloperoxidase and peroxinitrile from activated macrophages or neutrophils (740, 996, 997). Thus myeloperoxidase-derived HOCl (hypochlorous acid) results in tyrosine radical formation and tyrosine cross-linking or di-tyrosine formation (tyrosylation), as well as characteristic patterns of chlorinated tyrosine residues. Peroxynitrite derived from iNOS and ROS from myeloperoxidase in activated macrophages or neutrophils also results in characteristic tyrosine modifications. These molecular signatures are found in abundance in LDL from advanced human plaques, whereas modifications characteristic of metal-catalyzed oxidation are not (740, 996, 997). Furthermore, myeloperoxidase-generated reactive nitrogen species convert LDL into a form avidly taken up by macrophages with conversion to foam cells (1427).

The question then arises whether preformed oxLDL activates endothelium to summon the macrophages and/or neutrophils initially or whether oxLDL are formed mainly after macrophages and neutrophils have arrived and become activated. Yet even if formation of tyrosylated, chlorinated, or nitrated oxLDL requires the presence of activated macrophages or neutrophils, once formed these modified LDL could then perpetuate endothelial activation and otherwise promote atherosclerosis (1303, 1304). Unfortunately, the near absence of myeloperoxidase in experimental mouse atherosclerotic lesions will make clarification difficult, although humans with myeloperoxidase deficiency are apparently protected from atherosclerosis (1303).

The decrease in atherosclerosis seen after immunization of animals with various forms of oxidized LDL or malondialdehyde-treated LDL (148, 547, 1371) has been forwarded as an argument in support of the LDL oxidation hypothesis. However, immunization with oxLDL and other antigens can have mixed effects, including stimulating production of protective “natural IgM” or promotion of immune tolerance by activation of regulatory T-cells (see sect. VIC, 5 and 6). Given these considerations, there remains a need to explain how the endothelium is activated to attract the monocytes and neutrophils in the first place.

Aside from oxLDL, endothelial and VSMC can mildly oxidize LDL in culture and such “minimally modified” LDL (mmLDL) can also activate endothelial cells and initiate monocyte adherence and transmigration. These events were prevented by the presence of HDL or antioxidants (1289). In these studies, monocyte rather than neutrophil adhesion and migration seemed to be stimulated. Hypothetically, LDL trapped in the subendothelial space would be exposed to sufficient ROS from activated endothelial cells to result in such minimally oxidized LDL. Formation of oxidized LDL in the plasma is thought to be unlikely due to potent antioxidant factors there. And, some have questioned whether even mmLDL are formed in vivo in the surprisingly antioxidant-rich intima (1686). However, the observations in human fetuses cited above would suggest at least some oxidation is possible and occurs prior to the arrival of macrophages. Thus there might be microenvironments in the intima or surrounding ECs and VSMCs that might be depleted of hydrophilic (vitamin C, urate, and bilirubin), hydrophobic (vitamin E) and enzymatic (e.g., PON-1) antioxidants.
## Table 4. Endothelial activation by hyperlipidemia, other risk factors (20 genes tested)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Model</th>
<th>%Δ A</th>
<th>Function, Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR1</td>
<td>TLR1−/− LDLR−/−</td>
<td>NS</td>
<td>TLR1 acts as a coreceptor to TLR2. No change with high-fat diet feeding (endogenous ligand), but deficiency markedly reduces the excess abdominal atherosclerosis induced by exogenous ligand Pam3 (363).</td>
</tr>
<tr>
<td>TLR2</td>
<td>TLR2−/− LDLR−/−</td>
<td>↓ 32%</td>
<td>Clearly involved in endothelial activation. Upregulated in areas of disturbed flow while TLR4 is not. BMT from TLR2−/− had a lesser effect on atherosclerosis (1248).</td>
</tr>
<tr>
<td>TLR4</td>
<td>TLR4−/− apoE−/−</td>
<td>↓ 55%</td>
<td>Involved in endothelial cell activation by mnlDL as well as macrophage activation. Percent reduction in aortic sinus given (1207). Recent study confirms greater effect for TLR4 while TLR2 also significant (784). A prior study showed no effect of a TLR4 defective mutation (1983).</td>
</tr>
<tr>
<td>TLR6</td>
<td>TLR6−/− LDLR−/−</td>
<td>NS</td>
<td>TLR6 acts as a co-receptor to TLR2. Results similar to TLR1 KO but exogenous ligand was MALP2 (363).</td>
</tr>
<tr>
<td>RP105</td>
<td>RP105−/− BMT in LDLR−/−</td>
<td>↓ 43%</td>
<td>RP105 (radioprotective 105, also known as CD180) is a TLR4 homologue that forms homodimers and then forms a complex with TLR4 homodimers. TLR4 binds the LPS-binding protein MD2 while RP105 binds MD1 (2043). RP105 facilitates LPS recognition in B cells but also facilitates recognition of other microbial lipoproteins in macrophages (160). Decreased atherosclerosis also seen with hematopoetic deficiency (883), together with reduced splenic B cell proliferation and a marked reduction in intimal T cells.</td>
</tr>
<tr>
<td>CD14</td>
<td>CD14−/− apoE−/−</td>
<td>NS</td>
<td>Lack of effect suggests this LPS recognizing, transmembrane adapter, which is part of the TLR4 complex, does not influence atherogenesis (156).</td>
</tr>
<tr>
<td>TRIF</td>
<td>TRIF−/− LPS2−/− LDLR−/−</td>
<td>↓ 21%</td>
<td>The LPS2 mutation causes a truncated and nonfunctional TRIF protein. Significant reduction in atherosclerosis seen only after 15 wk of high-fat diet (1484).</td>
</tr>
<tr>
<td>LOX-1 (OLR1)</td>
<td>OLR1−/− LDLR−/−</td>
<td>↓ 50%</td>
<td>Involved in endothelial cell activation by oxLDL and TGLR as well as macrophage activation (1185).</td>
</tr>
<tr>
<td>RAGE</td>
<td>RAGE−/− apoE−/− DN-RAGE apoE−/−</td>
<td>↓ 52%</td>
<td>↓ 78%</td>
</tr>
<tr>
<td>AGE-R3</td>
<td>LGALS3−/− in fat-fed C57BL/6J</td>
<td>↑ 55%</td>
<td>LGALS3 (lectin, galactoside-binding, soluble, 3) is the preferred gene name for galectin 3, the alternative name of AGE-R3. AGE-R3 is considered a &quot;clearance receptor&quot; for AGE. Increased Th1 cells also seen in lesions (813). Conflicting results in apo E KO model (1266).</td>
</tr>
<tr>
<td>AR</td>
<td>AR−/− apoE−/−</td>
<td>↑ 180%</td>
<td>Although AR (aldose reductase, also known as AKR1B1 for aldo-keto reductase family 1, member B1) converts glucose to sorbitol in the polyol pathway, it also detoxifies reactive lipid aldehydes and precursors of AGE. Shown are data for nondiabetic mice whose arteries accumulated 4-hydroxy-trans-2-nonenal (HNE) (1671). Increased plasma HNE and hexanal were seen as well. In diabetic, apo E null mice, increase in atherosclerosis was similar while glyoxal-lysine adducts were particularly increased (90). Greatest effect in both models was on early atherosclerosis.</td>
</tr>
<tr>
<td></td>
<td>Human AR Tg in diabetic LDLR−/−</td>
<td>↑ 15%</td>
<td>Overexpression of human AR increased atherosclerosis only in diabetic mice (not normoglycemic LDLR−/−), presumably due to depletion of NADPH and impaired glutathione regeneration (1879). Diabetes itself increased lesion size by 93% overall compared with nondiabetic mice.</td>
</tr>
<tr>
<td>G2A (GPR132)</td>
<td>G2A−/− LDLR−/−</td>
<td>↓ 43%</td>
<td>G2A is a GPCR that recognizes LPC and LPS as well as certain oxidized fatty acids. The reduction in atherosclerosis was seen after Western-type diet feeding (1386). Essentially opposite effects on endothelial inflammatory signaling were seen with G2A KO in normolipidemic mice (174). In apo E-null mice, G2A deficiency in macrophages actually increased atherosclerosis, suggesting a modulating role specifically in macrophages and possibly in the absence of apo E (171). G2A signaling in macrophages appears to enhance efferocytosis (542). Further conflicting results have been published (1387).</td>
</tr>
<tr>
<td>LPA1/3</td>
<td>LPA1, LPA3 specific Abs in apoE−/−</td>
<td>↓ 40%</td>
<td>LPA administration also increased atherosclerosis in these mice (2110).</td>
</tr>
</tbody>
</table>

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Continued
With regard to the mechanism whereby mmLDL might activate endothelial cells, several investigators have suggested that arachidonic acid in the sn-2 position of phospholipids in trapped LDL becomes oxidized, resulting in abnormal phospholipids with structures similar to PAF (1288). This simplistic model is complicated, however, by findings that anti-inflammatory electrophilic compounds are also formed during LDL oxidation and that the above PAF-like lipids probably do not act through the PAF receptor (131, 196, 871).

B. TLR2 and TLR4

Toll-like receptors are among the most fundamental and best studied components of the innate immune system. The 10 known human TLRs recognize a host of exogenous PAMPs as well as endogenous DAMPs. TLR4 signaling is illustrated in FIGURE 13. TLR2 employs the same MyD88 adapter and downstream signaling components as shown in FIGURE 13 for TLR4 but lacks binding domains for TRIF or TRAM. Indeed, TLR4 is unique among TLRs in being able to mediate both MyD88-dependent NF-κB signaling as well as binding TRIF/TRAM adapters to activate interferon regulatory factor (IRF)3 and IRF7 transcription factors and IFN-β production (apparently a delayed feature which becomes active after TLR4 uptake into endosomes). The activation of IRF3/7 is analogous to the NEMO-IKK1/2 complex activation of NF-κB (284). Upon binding to TRIF, TRAF3 acts as an adapter to recruit E3 ubiquitin ligases (including Mib1/2) as well as a complex consisting of the scaffold TRAF family member-associated NF-κB activator (TANK) and IKK-related kinases, IKKe or TANK-binding kinase 1 (TBK1). Other scaffolds, NAP1 (NF-κB activating kinase-associated protein 1) and SINTBAD (similar to NAP1 TBK1 adapter) can act like TANK to bind IKKe or TBK1. K63 ubiquitination of TANK and TBK1 are involved in activation of TBK1, while IKKe may be activated somewhat differently (1032). Such ubiquitination may allow cross-talk between the IRF3/7 and NF-κB activation pathways. Once activated, IKKe and TBK1 serine phosphorylate IRF3 or IRF7 causing their activation and nuclear translocation, followed by transcription of the type I interferon IFN-β (which also requires binding of NF-κB and ATF2 to other domains of the promoter). Interest in this pathway has recently been piqued with findings that knock-out of IKKe or chemical inhibition of IKKe and TBK1 elevates energy expenditure and results in resistance to obesity or weight loss in fat-fed mice (1470).
TLR2 forms heterodimers with TLR1 or TLR6, whereas TLR4 typically acts as a homodimer. TLR2 can also utilize the scavenger receptor CD36 as a coreceptor. Classical exogenous PAMPs recognized by these receptors include LPS for TLR4 and bacterial lipoproteins or peptidoglycan for TLR2. Endogenous molecular patterns recognized by TLRs include certain oxidized lipids as found in oxLDL and mmLDL. Oxidized or otherwise altered phospholipids can also mark cells by protruding abnormally (much like whiskers) from the cell membrane and trigger innate immune receptors including TLR. Oxidized phospholipids also bind natural antibodies that act to clear the cells or particles displaying these damaged phospholipids (657, 1210). Such clearing activity may be more relevant to macrophage function rather than endothelial activation. Another endogenous ligand for TLR4 and TLR2 is the extracellular domain A (EDA) of fibronectin, whose expression is increased with aging (1563).

Both TLR2 and TLR4 are present on endothelial cells and can mediate inflammatory signaling. For example, both receptors were shown to induce endothelial expression and secretion of bone morphogenic protein 2 (BMP2) through NF-κB and ERK1/2 activation after exposure to oxLDL (1692). TLR4 binds LPS (and some other ligands) by way of the coreceptor CD14 after LPS is bound in serum to the acute phase reactant LPS binding protein (LBP, produced in liver). The LPS is then presented to the extracellular adapter MD2 (also known as lymphocyte antigen LY96) to facilitate TLR4 signaling. CD14 is expressed by human endothelial cells but at considerably lower levels than in monocytes, yet it is upregulated in endothelial cells by LPS as well as LPA (849).

There is considerable cross-talk between the TLR receptors. Activation of TLR4 can induce increased expression of TLR2 in endothelial cells (495). Inflammatory signaling through both TLR2 and TLR4 is increased in endothelial cells from type I diabetics without increased TLR2 or TLR4 protein levels, apparently due to greater expression of NF-κB (1024). Nevertheless, TLR2 is often thought to be more important for endothelial cell activation in atherogenesis while TLR4 appears to play a major role in macrophages and VSMC.

Evidence for a specific role in atherogenesis for TLR2 is plentiful. Whole body deficiency of TLR2 in high-fat fed LDLR KO mice reduced atherosclerosis significantly at 10 wk by ~50%, whereas transplant of TLR2-deficient bone marrow had no effect on atherosclerosis (1248). Nevertheless, if TLR2-deficient mice transplanted with TLR2+/+ bone marrow were exposed to an artificial TLR2 ligand, then atherosclerosis was markedly increased. In another study, when fed normal chow diet, TLR2 deficiency clearly reduced atherosclerosis in whole body KO (by 65–70% at 7 mo) (1062) as well as in mice transplanted with bone marrow deficient in TLR2 (731). Localized application of a synthetic TLR2-specific ligand to the femoral arteries of ApoE KO mice increased atherosclerosis at that location (1564). Importantly, endothelial expression of TLR2 was increased in areas of disturbed flow while that of TLR4 was not (1247). Furthermore, TLR2 blocking antibodies had a greater effect in mixed cell cultures from human carotid plaques to decrease NF-κB, MCP-1, and MMP production compared with blocking TLR4 (1226).

In contrast to the above observations regarding TLR2, whole body TLR4 KO in ApoE-deficient mice on a high-fat diet appeared to have less of an effect (24% reduction in atherosclerosis) (1207), or even no effect in another study (1983). However, yet another study reported a 50% reduction in atherosclerosis in C3H mice with ApoE deficiency fed a high-fat diet. C3H mice have a point mutation in the TIR domain of the cytoplasmic tail of TLR4 which blocks MyD88 binding and downstream signaling. In the same report, BMT studies showed a profound effect for vessel wall TLR4 expression when C57BL/6J or C3H mice (without ApoE deficiency) were fed a high-fat diet with cholate (1617). Importantly, the C3H endothelial cells were found to be unresponsive to mmLDL while exposing wild-type C57BL/6J endothelial cells to mmLDL caused marked activation. Interestingly, TRIF-defective LDLR KO mice had reduced atherosclerosis (1484), demonstrating that even if TLR4 receptors in C3H continued to signal through TRIF, they should not become protective. The possibility might have been considered reasonable considering TLR3, which signals only through TRIF, was found to be mildly protective (339, 1484). Macrophage accumulation and cytokine production in early plaques was reduced to a greater extent in TLR4 than TLR2 deficiency in a study that directly compared both in chow-fed ApoE-deficient mice. VSMC accumulation of lipid and secretion of MCP-1 was strongly dependent on TLR4 in the same study (764).

While several of the studies cited above, particularly for TLR4 and TLR3, seem most relevant to macrophage and VSMC activation, they remain relevant to the more general theme of initiation through activation of the innate immune response by hyperlipidemia and may apply to endothelial activation as well. Further research regarding TLR4 signaling in endothelial cells would be necessary to confirm this generalization. Nevertheless, TLR activation by hyperlipidemia in general is discussed here in a single section. Of considerable importance in this regard is the role of mmLDL in activating TLR4. In macrophages, mmLDL transmitted signals via TLR4 after specific binding to CD14 (1211), whereas for oxidized phosphatidylcholine, a different TLR4 adapter appeared to be involved (1892). Mono- and polyoxidized cholesterol arachidonate was found to be a key component of mmLDL that mediated TLR4 activation. Such oxidized cholesterol esters could be generated by exposure to 12/15-LO (721). In addition, mmLDL led to
TLR4 tyrosine phosphorylation, binding and activation of Syk, with downstream activation of the GEF, Vav1 which can activate Ras, Rac1, Rho, and Cdc42. Other effectors including PLCγ and PI3K are also activated. These led to macrophage cell membrane ruffling and promoted NOX2, ERK1/2, and JNK activation and associated proinflammatory signaling (318, 319, 1210).

Saturated fatty acids have been reported to activate TLR4 while polyunsaturated fatty acids, particularly n-3 appear inhibitory (1088). However, further study suggests that palmitate and to a lesser extent, stearate, rather than binding directly to TLR4, are converted to ceramide with activation of PKCζ, ERK1/2, JNK, and p38 which then act to amplify downstream TLR4 responses to LPS by threefold (as measured by IL-6 and IL-8 production) (1574). Incubation of aortic explants from wild-type and TLR4 KO mice with palmitate for 6 h also led to TLR4-dependent activation of PYK2 (890).

ECSIT (evolutionary conserved signaling intermediate in Toll pathways) is importantly involved in TLR signaling through effects mediated by TRAF6. ECSIT is thought to act as an adapter to directly promote MEK1 activation. More recently, ECSIT was recognized as a component of mitochondrial electron transport complex I. TRAF6 can ubiquitinate ECSIT, the two forming a complex on mitochondria which can promote association with phagosomes and greatly increase mitochondrial superoxide production (1945). Its relationship with atherosclerosis is apparently unknown.

C. Role of the Oxidized LDL Receptor 1

Early studies with oxLDL demonstrated powerful proinflammatory and apoptotic effects when incubated with endothelial cells. Subsequent studies identified lectin-like oxidized LDL receptor 1 (LOX-1), coded by the OLR1 (oxidized LDL receptor 1) gene, as a scavenger-type receptor expressed on the surface of endothelial cells which likely mediated these effects (1545). LOX-1 is also expressed on monocyte/macrophages and platelets, but unlike macrophages, LOX-1 is the only scavenger-type receptor expressed highly on endothelial cells. LOX-1 is yet another PAMP-recognizing receptor with a key role in innate immunity (728), being activated by certain gram-positive and gram-negative bacteria, apoptotic bodies, senescent red blood cells, activated white blood cells (WBC) and platelets, advanced glycation end-products (AGEs), lyssolecithin, and C-reactive protein (CRP) (561, 1621). Note, however, that transgenic CRP overexpression studies and a recently reported CRP knockout have failed to demonstrate any proatherogenic effect for CRP (see Table 9) (1778), consistent with the lack of causal effects in human “Mendelian randomization” studies (474).

Importantly, LOX-1 is not expressed constitutively but is induced by most of its ligands, including oxLDL, as well as factors which upregulate NADPH oxidase, such as ANG II. LOX-1 is strongly expressed on endothelial cells over human atherosclerotic plaque or associated vaso-vasorum as well as other cells in plaques (1184). LOX-1 expression is increased by high-cholesterol diet in ApoE KO mice, in hyperlipidemic rabbits, diabetic rats, hypertensive rats, and after exposure to various cytokines, particularly TNF-α (1020, 1182, 1184) as well as acute, but apparently not chronic laminar shear stress (403, 1255).

More complete understanding of LOX-1-mediated intracellular signaling has been frustrated by the lack of usual cytoplasmic domains (1081). Some of the identified pathways are depicted in Figure 13. Octamer binding transcription factor 1 (Oct1) is an apparently proatherogenic transcription factor activated by LOX-1. PKCα activation appears key in activating Oct1 (1792). Overlapping DNA binding sites between Oct1 and Nrf2 may lead to suppression of antioxidant responses generally (627). Oct1 also appears to repress several CYP enzymes (CYP1A1, CYP2A6/7, CYP2B6, CYP2C8, CYP2C9, CYP2J2) which can synthesize vasodilatory products such as 11,12-EEET. Oct1 may also be the means through which PKCα increases CD40 and CD40L expression (1019).

LOX-1 ligation also results in powerful proapoptotic and inflammatory responses due to inactivation of PI3K and PKB (1040, 1082). A major mechanism for these effects appears to be formation of a complex on the receptor cytoplasmic tail which includes ARHGEF1 and ROCK2. ARHGEF1 and MMP1 together activate RhoA (not found in the complex) which then activates ROCK2. Direct phosphorylation by ROCK2 inhibits eNOS and PI3K activity (1158). RhoA may also suppress PI3K through an effect on the actin cytoskeleton.

Serine phosphorylations of ROCK1 (and ROCK2) directly activate MLC while inhibiting MYPT1, leading to increased myosin phosphorylation, endothelial contraction, and opening of paracellular junctions to facilitate leukocyte transmigration. Importantly, ROCK2 is known to activate NF-κB in endothelial and other cells.

**FIGURE 13.** Activation of endothelial cells through LOX-1, RAGE, and TLR4 signaling. 1-Palmitoyl-2-epoxysiprostane-sn-glycero-3-phosphorylcholine (PEIPC) is one of several oxidized phospholipids that can activate TLR4. Comparable pathways are present in macrophages which can lead to activation and foam cell formation. CASP8, caspase-8; FN-EDA, fibronectin extra domain A, also termed EIIIA. FN-EDA is an alternatively spliced FN seen accumulating in artery walls and even plasma of aging mice which can bind and activate TLR4. See text for other abbreviations and additional explanation.
Thus LPA binding to its Ga13-coupled GPCR on endothelial cells activates a RhoGEF followed by RhoA activation of ROCK2. ROCK2 then phosphorylated its immediate substrates within 5 min, followed by peak Ser536 phosphorylation of the p65 subunit of NF-κB at 30 min and ICAM-1 and VCAM-1 expression within 4 h (1623).

LOX-1 activates Rac1 with a burst in NADPH oxidase activity by an unknown pathway (1694), but the increased ROS further activated JNK which promotes an oxidizing, proapoptotic milieu by stimulating ubiquitination and degradation of SOD2 (1739). The ROCK inhibitor fasudil led to a 54% reduction in atherosclerosis in ApoE-null mice (1984), confirming a prior observation using another ROCK inhibitor in LDLR KO mice which resulted in a 35% reduction in aortic sinus lesions (1128). Finally, activation of the Ras, Raf, ERK1/2 cascade leads to increase PAI-1 expression in response to oxLDL through the LOX-1 receptor (1536).

If oxLDL were the only lipoprotein to bind to the LOX-1 receptor, its relevance to early endothelial activation would not be so readily apparent for reasons noted above. However, in addition to oxLDL, LOX-1 is bound and strongly activated by electronegative LDL (1082). This finding is consistent with a substantial literature demonstrating the endothelial activating and apoptotic potential of electronegative LDL. These particles can be formed by a number of nonoxidative mechanisms as well as by oxidation (1535). Electronegative LDL are found in a much higher proportion of plasma LDL (up to 10% of normolipidemic LDL) than oxidized LDL. A variety of relevant mechanisms may be responsible for their formation. For example, in addition to oxidation, glycation, enrichment with nonesterified fatty acids, treatment with cholesteryl esterases, phospholipase A2, PAF-AH (platelet activating factor acyl hydrolase, also called LpPLA2), and, importantly, sphingomyelinase all lead to electronegative LDL (1348, 1535).

LDL themselves seem to carry a sphingomyelinase activity that promotes formation of electronegative LDL and that promotes LDL aggregation as seen in subendothelial, trapped LDL (103). Furthermore, sphingomyelinase and other enzymes capable of modifying LDL and promoting aggregation, are present in the artery wall and likely promote aggregation of LDL (1348).

LOX-1 can also mediate inflammatory responses to triglyceride-rich lipoproteins (TGRL) and their remnants (1382, 1626). Interestingly, pioglitazone, through PPARy, blocked LOX-1 signaling and the LOX-1 mediated increase in monocyte binding to endothelial cells (1185). Overexpression of LOX-1 in coronary arteries in ApoE KO mice leads to an atherosclerosis-like vasculopathy (825).

Other modification of LDL may also be sufficient for endothelial activation. Thus exposure of LDL to a proteinase and cholesteryl-esterase resulted in LDL that stimulated IL-8 release by incubated endothelial cells, an effect that the investigators attributed to free fatty acids (1716). Possibly, the treatment caused conversion of LDL to an electronegative LDL and activation of LOX-1. On the other hand, these findings may be consistent with recent studies demonstrating that exposure of endothelial cells to saturated fatty acids leads to a somewhat delayed activation of endothelial cells through a mechanism dependent on TLR4 and NADPH oxidase as well as downstream activation of NF-κB (1131). Feeding mice a diet high in saturated fat resulted in similar activation (905). Incubation with LDL at concentrations of 220 mg/dl (LDL-C), isolated with scrupulous care to avoid oxidation or other modification, also activates endothelial cells in culture (2114). However, whether such high concentrations may have included nonoxidatively modified LDL, such as electronegative LDL, is not clear.

Additional potential mechanisms for the atherogenicity of TGRL remnants have been reported. TGRL may activate endothelial cells or promote increased responsiveness to subsequent activation without any need for modification (2, 430, 1319). Apo CIII, which accumulates on TGRL, in some way appears to activate PKCβ and thereby inhibits protective Akt signaling in endothelial cells (891). Postprandial TGRL isolated after a cream meal (as compared with fasting TGRL) were particularly pro-inflammatory (1316). Apo CI, which inhibits removal of TGRL, elevates levels of these lipoproteins when over-expressed and increases atherosclerosis in ApoE-deficient mice (346), but pro-atherogenic alterations in TGRL composition with increased apo CI expression is also a possibility.

D. RAGE Is Activated by Hyperlipidemia

RAGE (receptor for advanced glycation end-products) is present on endothelial cells, and, like TLR4 and LOX-1, can activate endothelial cells by overlapping intracellular signaling pathways (see Figure 13). Knowledge of the actual transduction pathways through the cytoplasmic tail of RAGE remains incomplete, but receptor ligation by a number of RAGE ligands activates NF-κB, JNK, and the JAK-STAT3 pathway (1486).

RAGE is generally considered in the context of diabetes-related modifications to proteins or LDL. AGE accumulates as products of nonenzymatic reactions between glucose and
the lysine on proteins (yielding carboxy-methyl lysine adducts) or the more reactive methylglyoxal reacting with arginine (with the latter also found in tissues of fat-fed mice) (1803). Diabetes and glucose-derived AGE clearly contributes to endothelial activation and atherosclerosis (1275, 1660). However, hyperlipidemia without diabetes also generates a substantial load of ligands for the RAGE. Indeed, 52% reduction in atherosclerosis was seen in nondiabetic, ApoE KO mice that were also deficient in RAGE while those expressing endothelial cell-specific dominant-negative RAGE mutations had more than 70% reduction (718). Similar reductions were seen for RAGE-LDLR double KO mice (1711). Activated inflammatory cells in early lesions can release other ligands for RAGE including S100/calgranulins and high-mobility group box 1 (HMGB1) (718). Thus RAGE represents yet another pathway for endothelial cell activation.

Besides the inflammatory RAGE receptor, there are two AGE “clearance” receptors, AGE-R1 and AGE-R3 (1661). Both promote endocytosis and removal of AGE. Additionally, an important anti-inflammatory mechanism for AGE-R1 appears to be binding of EGFR and inhibition of EGFR transactivation in response to AGE exposure (238, 239). AGE exposure results in a marked increase in tyrosine phosphorylation status of the cytoplasmic tail of EGFR, possibly by generation of active Src and/or ROS and resultant inhibition of phosphatases. Activation of EGFR results in activation of PKCδ (through phosphorylation of tyrosines 311 and 332), phosphorylation and assembly of adapter proteins p66Shc, Grb2/Sos, subsequent activation of p47phox component of NOX, activation of Raf and the ERK MAPK pathway as well as activation of NF-kB. Activated PKCδ phosphorylates the p47phox component of NOX, promoting its translocation to the membrane, NOX activation, and further increased ROS production. AGE receptor 1 (AGE-R1) forms a complex with EGFR, binds AGE, and buffers or inhibits the activation of EGFR (239). PKCδ would also be expected to activate PYK2 with its numerous proinflammatory effects. Transgenic overexpression of AGE-R1-protected mice from diabetes induced by a high-fat diet and decreased intimal hyperplasia and inflammation caused by arterial wire injury in fat-fed animals (1803). AGE-R1 expression can be upregulated by its ligands but is repressed by prolonged exposure to pro-oxidant stress. Thus increased AGE-R1 expression was seen with a diet low in AGE which also was found to extend life in mice, while increased oxidant stress in diabetes or high AGE diet led eventually to downregulation of AGE-R1 (239, 1661).

AGE-R3 (also known as galectin-3) may serve as both an AGE clearance receptor and a mediator of macrophage endocytosis of modified LDL (1266). In fat-fed C57BL/6J mice, AGE-R3 deletion increased atherosclerosis and was associated with increased TH1 cell infiltration into lesions (813). However, after 36–44 wk in ApoE KO mice atherosclerosis was actually decreased in AGE-R3-deficient animals. However, there was a trend in the opposite direction in younger animals.

### E. LPC- and LPA-Mediated Signaling in Endothelial Activation

Generation of LPC in trapped LDL may be among the earliest relevant pathways of endothelial activation through activation of PYK2 (1488). LPC content of LDL is increased markedly by oxidation (with subsequent hydrolysis by PAF-AH), but LPC is also formed from nonoxidized precursor by sPLA2 activity. Which of these potential pathways is quantitatively most important is unknown. LPS may be similarly formed. LPC signaling resulted in PYK2 activation which was evident in endothelial cells of the aorta as soon as 7 days after starting a Western-type diet in ApoE-deficient mice (890). However, this study did not identify the particular GPCR that activated PYK2 by LPC, only that it was coupled to Gα1.

G2A (also known as GPR132 and having higher affinity) and GPR4 were reported as GPCR which were thought to recognize LPC and LPS as ligands. Certainly, LPC seems to mediate a great deal of proinflammatory signaling in a number of relevant cells, including endothelial cells (1156). But apparently, early evidence on LPC binding to G2A and GPR4 could not be reproduced, followed by retraction of the original articles (1156). Which GPCR mediates LPC induced PYK2 activation apparently remains unknown. One study reported that G2A was inhibited by LPC and that G2A is activated instead by an extracellular reduction in pH (1252). G2A deficiency has been reported to decrease atherosclerosis (1386) or increase it (171). Apparently, macrophage G2A is protective because normal G2A activity promotes early apoptosis of macrophages with less accumulation. Yet G2A also enhances effecrosis and a less inflammatory M2 phenotype (171).

Based on the observation that LPC is converted to LPA by endothelial extracellular ATX, perhaps the proinflammatory effects attributed to LPC can be largely attributed to LPA instead. Indeed, a great deal of interest has been devoted to development of ATX inhibitors but apparently effects on atherosclerosis have not yet been tested (42). Interest further intensified after the discovery of several (at least 6) LPA receptors (LPA1–6 encoded by genes LPAR1–5). LPA1–6 show variable and overlapping G protein coupling and some can activate PYK2 as well as the CBM complex (through β-arrestin2 binding) with NF-κB as a downstream target (426, 1383, 1708, 1714). Adenosine receptors P2Y5 and P2Y10 seem also to be LPA targets (316). Unsaturated LPA species have generally been found most active (316). LPA has long been known to both activate human platelets and to be released by activated platelets (1514, 1631). The platelet activating effect appears me-
LPA1/3 receptors are Gαq coupled, probably together with effects from other LPA receptors. However, in mouse, the predominant effect of LPA is inhibition of platelet aggregation by LPA4 (with Gαi coupling).

LPA accumulates in human atheroma (188) and promotes monocyte-platelet aggregates (1631). LPC is generated in mildly oxidized lipoproteins by the action of Lp-PLA₂ (lipoprotein-associated phospholipase A₂). LPA is synthesized from this LPC when endothelial cells or carotid arteries are incubated with mildly oxidized lipoproteins. LPA was recently shown to stimulate elaboration of the cytokine CXCL1 (also known as GROα), as well as stimulate Weibel-Palade body exocytosis with expression of P-selectin on endothelial cells resulting in enhanced monocyte rolling and capture. CXCL1 is also important for neutrophil capture and migration. These effects are apparently mediated by LPA1 and LPA3 which can both be coupled to G_{i/0} or G_{q/11} with LPA1 additionally coupled to G_{12/13} (316). PYK2 signaling is also activated. Treatment of ApoE-deficient mice with LPA (with arachidonate as the fatty acid) approximately doubled atherosclerosis while an inhibitor currently being tested in larger phase III clinical trials (1594). A more direct approach may prove to be blocking LPA formation by inhibiting ATX or development of specific LPA receptor inhibitors.

F. Redundancy in Endothelial Activation Pathways

Perhaps the major message behind Figure 13 and the above discussion is the redundancy of pathways potentially leading to endothelial cell activation. Modified lipoproteins may activate endothelial cells by way of TLR2, TLR4, LOX-1, RAGE, or LPA1/3. The TLR4 and RAGE responses may require oxidative changes, as found in mmLDL or oxidized phospholipids, but may also be triggered by saturated fatty acids (for TLR4), while the LOX-1 response appears to include a broader array of triggers.

An intervention that focuses on only one of these redundant proinflammatory endothelial pathways, such as oral antioxidants (even acknowledging the limitations of the antioxidants tested thus far), might be expected to be ineffectual in reducing coronary events. This holds even if lipoprotein oxidation was causal. Redundancy seems to be the rule for pathways vital to the survival of the organism. For the endothelium, the key roles that various forms of activation play in defense against microorganisms, repair or angiogenesis, as well as in clearing the bloodstream of senescent or apoptotic debris all seems self-evident. In this sense, it may be disingenuous to describe the state of endothelial activation associated with dyslipidemia as “dysfunction.” Indeed, the cells are likely performing admirably well and as expected in response to perceived threats.

VI. INITIATION OF ATHEROSCLEROSIS: LEUKOCYTE TRANSMIGRATION AND ACTIVATION

A. Purinergic Signaling

ATP, ADP, and adenosine are among the most ancient and widely utilized extracellular signaling molecules. The relatively high ATP concentrations in living, viable cells (1,000-fold higher inside RBC versus plasma) makes ATP a useful “alarm” or “danger” signal when found at higher levels outside of cells. ATP can act as a relatively short range chemoattractant, a “find me” signal marking apoptotic cells for phagocytosis (473), or stimulate endothelial cell migration (759). ATP is released from vesicles as a cotransmitter by numerous neurons (235). It can potentiate acetylcholine-induced muscle contraction, mediate nociception, control O₂ sensing in the carotid body, and activate tubuloglomerular feedback in the kidney. ATP is hydrolyzed to ADP, AMP, and adenosine by extracellular ectonucleotidases.

In migrating phagocytes, release of ATP through connexin (Cx) or pannexin (Px) hemichannels at the leading edge serves in an autocrine fashion to amplify chemotactrant signals (955). Between adjacent cells, a connexin hemichannel on each cell joins to form a gap junction with the capability of transmitting between cells electrical, calcium, or other chemical signals relevant to purinergic signaling. Finally, a series of purinergic receptors have been defined that are activated by ATP, UTP, ADP, UDP, or adenosine. They include four P1 family GPCR (metabotropic receptors) activated by adenosine (A₁R, A₂AR, A₂BR, A₃R), seven P2X family ligand-gated cation channels (ionotropic receptors) which only respond to ATP, and eight P2Y GPCR. A₂AR antagonism appears to be caffeine’s primary mechanism for mediating alertness (797), although caffeine also has affinity for A₁R and heterodimers of the two receptors may better reflect complex effects and caffeine tolerance (524).
(P2Y₁, P2Y₁₂) that trigger platelet shape change and aggregation, respectively. The widely used antiplatelet thienopyridine drug clopidogrel was developed before the purinergic receptors were recognized but is now known to irreversibly bind and inhibit the P2Y₁₂ receptor. Newer thienopyridines, prasugrel and ticagrelor, are also P2Y₁₂ blockers. The remaining purine receptors represent a rich and relatively untapped source of pharmacological opportunities for cardiovascular intervention (486).

The relevance of purinergic signaling to atherosclerosis has been illustrated by a series of genetic mouse models (1551). Monocyte Cx37 serves as a hemichannel for release of ATP (see FIGURE 14). ATP and ADP purinergic signaling is pro-inflammatory (probably through endothelial P2Y₁, P2Y₂, or P2X receptors), even though these receptors also mediate rapid ATP-induced vasodilation (1059, 1596, 1646). Nevertheless, Cx37 KO mice had increased atherosclerosis (see TABLE 5). The effect was entirely due to leukocyte release of ATP, since endothelial-specific Cx37 KO had no effect on atherosclerosis (1974). This protective effect of ATP appears to be due to rapid conversion of ATP and ADP to adenosine by the sequential action of the two endothelial cell-surface ectonucleotidase enzymes CD39 and CD73. KO of either of these ectonucleotidases increased atherosclerosis (see TABLE 5). CD73-deficient endothelial cells express more adhesion molecules (665).

Anti-inflammatory signaling by adenosine may largely be through A₂BₐR, with upregulation of CD73 expression on the endothelial cell surface as well (1551). Deletion of A₂BₐR increased catheter-induced intimal VSMC hyperplasia, together with increased TNF-α expression, effects probably mediated by bone marrow-derived platelets (2019); yet increased expression of adhesion molecules was seen on endothelium of whole body A₂BₐR KO mice (16). A₂BₐR activation increases cAMP, which is generally anti-inflammatory and also specifically downregulates expression P2Y₁ ADP receptors in platelets (2018).

In contrast to generally anti-inflammatory A₂BₐR signaling, whole body A₂AₐR deficiency resulted in a 24% reduction of atherosclerosis largely due to a marked reduction in macrophage numbers. Plaques, although smaller, displayed markers of greater inflammation, with impaired PKA activity (1898). Other effects of A₂AₐR signaling include increased ABCA1 transporter expression and cholesterol efflux (149) and increased ANG II-induced NOX2 activation (1779).

![FIGURE 14. Examples of purinergic signaling. CD39 is also known as NTPDase1 (nucleoside triphosphate diphosphohydrolase 1). Endothelial P2Y receptors are Gq coupled and stimulate PLC and IP3 release, which activates IP3R to promote release of ER-stored calcium. Calcium release activates cPLA2 with liberation of free arachidonate followed by prostacyclin and EET synthesis and secretion. Px1, pannexin 1.](http://physrev.physiology.org/Downloadedfromhttp://physrev.physiology.org/)
Cx homodimers form gap junctions between cells and can transmit either pro- or anti-inflammatory signals, depending on the specific substrates transported by the channel (such as electrical currents, ionized calcium, and even cAMP). Permeability can be controlled by various intracellular signaling molecules. Cx40 may allow anti-inflammatory cAMP to diffuse between cells. Endothelial Cx40 is widely expressed in the endothelium of mice and rats (268, 572) except in cells covering advanced atherosclerotic plaques (268). Whole body Cx40 deficiency causes hypertension due to renal effects. Endothelial-specific Cx40 KO decreased atherosclerosis in ApoE-deficient mice. Interestingly, the effect was due entirely to vessel wall expression, rather than hematopoetic deficiency of A2AR KO showed modest reduction in plaques. More macrophage apoptosis and greater inflammation seen (1898). Net decrease in plaque even though A2AR signaling upregulates ABCA1 and inhibits IFN-γ signaling in macrophages (1884).

### TABLE 5. Purinergic signaling in endothelial and other cells (9 genes tested)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Model</th>
<th>%ΔA</th>
<th>Function, Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cx37</td>
<td>Cx37&lt;sup&gt;-/-&lt;/sup&gt; BMT into apoE&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>↑ 58%</td>
<td>Monocyte Cx37 serves as a hemichannel for release of ATP to endothelium. Cx37 KO monocytes showed enhanced recruitment to atherosclerotic lesions. Endothelium-specific Cx37 KO had no effect on atherosclerosis (1974).</td>
</tr>
<tr>
<td>CD39</td>
<td>CD39&lt;sup&gt;+/+&lt;/sup&gt; apoE&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>↑ 108%</td>
<td>CD39 is an ectonucleotidase, converting ATP and ADP to AMP. The heterozygous KO had a greater increase in plaque than the double KO (just 33% increase) possible due to paradoxical S1P decrease in platelet activity in the double KO (812).</td>
</tr>
<tr>
<td>CD73</td>
<td>CD73&lt;sup&gt;-/-&lt;/sup&gt; apoE&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>↑ 150%</td>
<td>CD73 is another ectonucleotidase. It converts AMP to adenosine (195).</td>
</tr>
<tr>
<td>Cx43</td>
<td>endothelial-specific Cre-Lox Cx43&lt;sup&gt;+/+&lt;/sup&gt; in apoE&lt;sup&gt;-/-&lt;/sup&gt; mice</td>
<td>↑ 117%</td>
<td>Also known as GJA5 (gap junction protein alpha-5). Shown here is effect on lesion area in thoracoabdominal aorta. No effect seen in aortic sinus except very early lesions in chow-fed mice. Endothelial adhesion molecules were increased. Whole body Cx40 deficiency causes hypertension which was avoided by endothelial-specific expression (268).</td>
</tr>
<tr>
<td>Cx43</td>
<td>Cx43&lt;sup&gt;-/-&lt;/sup&gt; LDLR&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>↓ 50%</td>
<td>Cx43 is upregulated in athero-prone areas and by onset of shear stress. Heterozygous KO also had decreased inflammatory cell accumulation (967).</td>
</tr>
<tr>
<td>A&lt;sub&gt;2&lt;/sub&gt;A&lt;sub&gt;R&lt;/sub&gt;</td>
<td>A&lt;sub&gt;2&lt;/sub&gt;A&lt;sub&gt;R&lt;sup&gt;+/&lt;/sup&gt;&lt;/sub&gt; apoE&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>↓ 32%</td>
<td>Both whole body and hematopoietic deficiency of A&lt;sub&gt;2&lt;/sub&gt;A&lt;sub&gt;R&lt;/sub&gt; KO showed modest reduction in plaques. More macrophage apoptosis and greater inflammation seen (1898). Net decrease in plaque even though A&lt;sub&gt;2&lt;/sub&gt;A&lt;sub&gt;R&lt;/sub&gt; signaling upregulates ABCA1 and inhibits IFN-γ signaling in macrophages (1884).</td>
</tr>
<tr>
<td>A&lt;sub&gt;2&lt;/sub&gt;B&lt;sub&gt;R&lt;/sub&gt;</td>
<td>A&lt;sub&gt;2&lt;/sub&gt;B&lt;sub&gt;R&lt;sup&gt;+/&lt;/sup&gt;&lt;/sub&gt; apoE&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>↑ 67%</td>
<td>Increased atherosclerosis due to worse hyperlipidemia in these mice (data for 16 wk shown). BMT of A&lt;sub&gt;2&lt;/sub&gt;B&lt;sub&gt;R&lt;sup&gt;-/-&lt;/sup&gt;&lt;/sub&gt; apoE&lt;sup&gt;-/-&lt;/sup&gt; marrow did not cause hyperlipidemia or increase atherosclerosis. Hepatic A&lt;sub&gt;2&lt;/sub&gt;B&lt;sub&gt;R&lt;/sub&gt;-derived cAMP normally suppresses SREB1c. Unclear if an additional direct effect on artery wall is present (941). Larger lesions with increased chronic TNF-α expression after catheter injury seen in A&lt;sub&gt;2&lt;/sub&gt;B&lt;sub&gt;R&lt;sup&gt;-/-&lt;/sup&gt;&lt;/sub&gt; mice (2019).</td>
</tr>
<tr>
<td>P2Y&lt;sub&gt;1&lt;/sub&gt;</td>
<td>P2Y&lt;sub&gt;1&lt;sup&gt;-&lt;/sup&gt;&lt;/sub&gt; apoE&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>↓ 24%</td>
<td>Effect due entirely to endothelial/VSMC expression, not platelets or other leukocytes based on BMT experiments (741).</td>
</tr>
<tr>
<td>P2Y&lt;sub&gt;12&lt;/sub&gt;</td>
<td>P2Y&lt;sub&gt;12&lt;sup&gt;-&lt;/sup&gt;&lt;/sub&gt; in C57BL/6</td>
<td>—</td>
<td>Less intimal proliferation in endothelial injury models. BMT studies suggested importance of platelet P2Y&lt;sub&gt;12&lt;/sub&gt; and inhibition with clopidogrel (490).</td>
</tr>
</tbody>
</table>

Reference numbers are given in parentheses.

Potential effects of purinergic P2 receptors are only beginning to be explored using genetic mouse models (see TABLE 5). P2Y<sub>1</sub> deficiency modestly decreased atherosclerosis in ApoE-deficient mice. Interestingly, the effect was due entirely to vessel wall expression, rather than hematopoetic cells including platelets (741). P2Y<sub>1</sub> signaling upregulates eNOS in endothelial cells, but the intermediate pathways are not well understood (372, 759). As P2Y<sub>1</sub> is G<sub>q</sub>-coupled receptor, which one might expect activation of PYK2 and subsequent ERK1/2 activation as the explanation for eNOS activation together with an overall proatherogenic effect. P2Y<sub>12</sub>, although prothrombotic, had a neutral effect on atherosclerosis (see TABLE 5). The P2Y<sub>6</sub> receptor, a G<sub>q</sub>-coupled receptor activated by UDP, seems to be important in promoting macropage accumulation and activation in atherosclerotic plaques and selective pharmacological inhibition reduced atherosclerosis in ApoE KO mice (673).
One additional aspect of purinergic signaling is illustrated in Figure 14, the role of red blood cells in controlling tissue blood flow. This is of particular interest to investigators performing studies of endothelial function based on flow-mediated dilation. The most commonly used methodology to stimulate brachial blood flow is to impose ischemia downstream of the brachial artery. Typically, a blood pressure cuff inflated well above systolic pressure is applied for 5 min, followed by sudden release of the cuff. Ischemia, imposed by the cuff, is known to cause vasodilation of resistance vessels. Release of the cuff then leads to rapid flow through the brachial artery. The increased flow stimulates endothelial-dependent vasodilation, typically monitored by brachial ultrasound. Surprisingly, vasodilation in ischemic tissue is dependent on the presence of red blood cells (RBC). This fundamental insight solves years of inquiries regarding how tissues match blood flow with oxygen demand (475, 486). In the face of hypoxia, Ga signaling (both α and α stimulate cAMP production through the adenylate cyclase type II found in RBC) leads to an increase in RBC cAMP. This increased cAMP triggers, in a complex fashion, release of ATP through the P2X1 hemichannel (1670). ATP has an acute vasodilatory effect which requires P2Y1 and P2Y2 ligation on endothelial cells as shown in Figure 14 (475). In addition, recent studies point to a major vasodilatory role for NO released by red cells. Remarkably, deoxymyoglobin acts as a catalyst to rapidly produce NO from nitrite in the face of hypoxia, as in exercising muscle (454). How these newly recognized pathways, together with other mechanisms of local control of blood flow such as myogenic responses (335), relate to long-term blood pressure control are questions that remain essentially unexplored and deserve careful attention, particularly with regard to “autoregulation” as referenced in the Guyton model of hypertension (6, 679, 928).

2. Chemokines induce high-affinity integrin binding and set the stage for transmigration

While partially tethered and rolling along the endothelial surface, the leukocyte brushes through the glycocalyx and encounters sequestered chemokines (chemotactic cytokines). Chemokines activate integrins to mediate firm adhesion and diapedesis and provide a concentration gradient to guide the direction of leukocyte movement. Systematic names classify chemokines by the cysteine spacing in their NH2 terminus, followed by an L for ligand or R receptor and a number. Thus CC refers to two consecutive cysteines; CXC is two cysteines with one intervening amino acid, etc. (1764). Among the most important of chemokines is CCL2 (commonly referred to as MCP-1). Some of the surface-immobilized chemokines noted above include CXCL1, CXCL2, CXCL4, and CCL5 in addition to MCP-1 (CCL2). CX3CL1 (fractalkine) is a membrane-bound chemokine. Some chemokines act to guide leukocytes into various areas of immune organs (lymph nodes, spleen) for further maturation. Various combinations of chemokines work to selectively recruit specific leukocyte subsets to the inflammatory site. Figure 15 depicts some chemokine-receptor pairs that help recruit monocytes, but several are shared by other leukocytes, particularly neutrophils (580, 2065). The combined deletion of CCL2, CCR5, and CX3CR1 almost entirely abrogated atherosclerosis in ApoE-deficient mice in proportion to the marked reduction in circulating monocytes (345).
Activated endothelial cells secrete CCL2 (MCP-1) which captures passing monocytes. T-cell receptor (TCR) recognition of antigens presented by endothelial cells can also incite T-cell integrin activation and T-cell capture. After transmigration and transformation, activated macrophages or foam cells themselves secrete MCP-1, providing a positive feedback signal for additional macrophage recruitment. Th1 cells express CCR5 and CXCR3 and adhere to activated endothelium expressing CCL5, CXCL9, CXCL10, and CXCL11 (without the need for TCR activation). Th1 cells enter plaque and secrete IFNγ which stimulates expression of CXCL10 by VSMC and CXCL9 and CXCL11 by macrophages and endothelial cells. Endothelium covering human atherosclerotic plaque was confirmed to express MCP-1, GROβ (CXCL1), and IL-8 (CXCL8), and these chemokines could induce attachment and spreading of test monocytes, demonstrating the relevance of prior, mostly mouse-based models (1379).

The GPCR which transduce the signals from chemokines are mostly coupled to G proteins of the Gαi type with some Gαq.
### Table 6. Ligands and corresponding cognate receptors in endothelial-leukocyte interactions (43 genes tested)

<table>
<thead>
<tr>
<th>Endothelium</th>
<th>Leukocyte</th>
<th>%Δ A</th>
<th>Model</th>
<th>%Δ A</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E-selectin (SELE)</strong></td>
<td>CD44-/− apoE-/− (360). CD44 also binds to hyaluronic acid and promotes leukocyte attachment to endothelium and helps regulate VSMC proliferation. Both bone marrow derived and endothelial or VSMC apparently contribute to atherosclerosis (2090).</td>
<td>↓ 70%</td>
<td>Tethering or rolling</td>
<td></td>
</tr>
<tr>
<td>P-selectin (SELP)</td>
<td>P-selectin-/− apoE-/− (343).</td>
<td>↓ 24%</td>
<td>ESL-1, PSGL-1, KN1A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P-selectin-/− LDLR-/− (assessed at 37 wk, NS) (862).</td>
<td>↓ 20%</td>
<td>PSGL-1 (CD24, CD34)</td>
<td>↓ 65%</td>
</tr>
<tr>
<td></td>
<td>P-selectin-/− apoE-/− (343).</td>
<td>↓ 45%</td>
<td>PSGL-1 (CD24, CD34)</td>
<td>↓ 65%</td>
</tr>
<tr>
<td></td>
<td>P-selectin-/− in C57BL/6 (1272). BMT confirms a modest role for platelet P-selectin (232)</td>
<td>↓ 45%</td>
<td>PSGL-1 (CD24, CD34)</td>
<td>↓ 65%</td>
</tr>
<tr>
<td></td>
<td>FucT-VII-/− BMT into LDLR-/− mice. Selectin ligands ESL-1 and PSGL-1 express glycans tipped with a fucosylated and sialylated tetrasaccharide which is critical for binding to the lectin domain of the selectins. Addition of fucose is mediated by (1,3)fucosyltransferases (FucT)-IV and –VII, also known as Fut7 (619).</td>
<td>↓ 34%</td>
<td>C2GlcNAcT-I/−/− apoE−/−</td>
<td>↓ 50%</td>
</tr>
<tr>
<td>PSGL-1 (CD24, CD34)</td>
<td>PSGL-1-/− apoE-/− (1089)</td>
<td>↓ 65%</td>
<td>L-selectin (on multiple cell types)</td>
<td>L-selectin−/− apoE−/− leads to reduced lymphocyte entry into aorta. No assessment of atherosclerosis (580, 581).</td>
</tr>
<tr>
<td></td>
<td>(see above)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CCL2 (MCP-1)</strong></td>
<td>CCR2−/− apoE−/− (1443).</td>
<td>↓ 78%</td>
<td>CCR2−/− apoE−/− (666).</td>
<td>↓ 62%</td>
</tr>
<tr>
<td></td>
<td>CCR2−/− apoE−/− (1443).</td>
<td>↓ 98%</td>
<td>CCR2−/− apoE−/− (179).</td>
<td>↓ 47%</td>
</tr>
<tr>
<td></td>
<td>CCR2−/− LDLR−/− (666).</td>
<td>↓ 79%</td>
<td>CCR2−/− apoE−/− (390).</td>
<td>↓ 67%</td>
</tr>
<tr>
<td></td>
<td>CCL2−/− in human apoB transgenic mice (644).</td>
<td>↓ 60%</td>
<td>CCR2−/− apoE Leiden (8 wk high-fat diet) (675).</td>
<td>↓ 86%</td>
</tr>
<tr>
<td>Note: MCP-2, 3, 4, and −5 (CCL8, 7, 13, and 12 respectively) also bind CCR2 in either mouse or human but direct effects on atherosclerosis have apparently not been studied.</td>
<td>CCR2−/− bone marrow transplanted to apoE−/− mice (1871).</td>
<td>↓ 62%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CCR2 expression and interaction with ligands MCP-1 and MCP-3 are critical for monocyte egress from bone marrow in response to inflammatory signals (1815).</td>
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</tbody>
</table>

**Activation (inside-out signaling to activate integrins) and chemotaxis by chemokines**

- CCL2−/− apoE−/− (1443).
- CCR2−/− apoE−/− (1443).
- CCR2−/− LDLR−/− (666).
- CCR2−/− in human apoB transgenic mice (644).

Continued
Table 6.—Continued

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>Model</th>
<th>%Δ A</th>
<th>Leukocyte</th>
<th>Model</th>
<th>%Δ A</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL3 (macrophages, activated platelets, neutrophils, and mast cells)</td>
<td>Also known as MIP-1α (macrophage inflammatory protein 1α). BMT of CCL3−/− marrow into LDLR−/−. Marked reduction of CCL3 seen (393)</td>
<td>↓ 31%</td>
<td>CCR1, 4, 5</td>
<td>CCR1−/− apoE−/− (205). CCR1 is found particularly on T-cells. See below for CCL5.</td>
<td></td>
</tr>
<tr>
<td>CCL5 (RANTES) (also in VSMC, platelets)</td>
<td>CCL5−/− apoE−/− early (965). NS. CCL5−/− apoE−/− late stage. Also decreased with BMT (1446). Another BMT study found less inflammatory lesions with more collagen early; no difference at 35 wk (1433). Also involved in activating medial VSMC to become lymphoid tissue organizers in adventitia (1077).</td>
<td>↓ 5%</td>
<td>CCR1, 3, 5</td>
<td>See above for CCR1. CCR5−/− ↓ 140% apoE−/− (205). Increase in endothelial progenitor cells seen. CCL5 can be deposited on endothelial cells from platelets and then cause neutrophil migration into early lesions dependent on CCR5. Neutrophil depletion decreased 4-week lesions ~50% but not later lesions (445).</td>
<td></td>
</tr>
<tr>
<td>CCL20 (activated VSMC)</td>
<td>Also known as MIP-3α (macrophage inflammatory protein 3α). Increased plasma levels in humans with hyperlipidemia and in atherosclerotic plaque. Increased expression by VSMC exposed to LDL, dependent on NF-B (245).</td>
<td>—</td>
<td>CCR6</td>
<td>CCR6−/− apoE−/−. Similar reduction with CCR6−/− BMT into apoE−/−. Reduction of macrophages in plaque paralleled reduced monocyte counts in blood. Required for macrophage mobilization from bone marrow (1894). Adoptive transfer of CCR6−/− B-cells into B-cell deficient (μMT) mice. CCR6 is also required for proper homing of B cells to aortic adventitia (437).</td>
<td></td>
</tr>
<tr>
<td>CCL19, CCL21 (activated VSMC)</td>
<td>Lymphorganogenic chemokines (also CXCL13). Secreted by cells in lymph nodes to promote homing of DC and naive T-cells (1077)</td>
<td>—</td>
<td>CCR7</td>
<td>Expressed by DC and naive T-cells to promote homing to lymph nodes. Also required for T-cell entry into inflammed plaque (1084).</td>
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</tr>
<tr>
<td>CXCL1 (GROα)</td>
<td>CXCL1−/− LDLR−/− (167). Effects primarily seen for macrophage accumulation in established fatty streaks, not earlier lesions.</td>
<td>↓ 22%</td>
<td>CXCR2 (neutrophils, mast cells, monocytes)</td>
<td>CXCR2−/− BMT into LDLR−/− (168).</td>
<td>↓ 62%</td>
</tr>
<tr>
<td>CXCL2 (GROβ)</td>
<td>—</td>
<td>CXCR2</td>
<td>see above</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CXCL3 (GROγ)</td>
<td>—</td>
<td>CXCR2</td>
<td>see above</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CXCL4 (platelet factor 4-in platelets)</td>
<td>PF4−/− in C57BL/6 (1521). PF4−/− apoE−/− (1521). CCL5 and CXCL4 can form heterodimers and promote monocyte adhesion to endothelial cells (2064).</td>
<td>↓ 80%</td>
<td>CXCR3B</td>
<td>splice variant of CXCR3 (see below)</td>
<td>—</td>
</tr>
<tr>
<td>CXCL8 (IL-8)</td>
<td>Stored in Weibel-Palade bodies in endothelial cell. Mediates both monocyte and neutrophil adhesion. KC [CXCL1 is often considered the mouse analog].</td>
<td>—</td>
<td>CXCR2</td>
<td>see above</td>
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Table 6.—Continued

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<tr>
<th>Endothelium</th>
<th>Model</th>
<th>%Δ A</th>
<th>Leukocyte</th>
<th>Model</th>
<th>%Δ A</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCL9,10,11</td>
<td>CXCL10&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt; (745). Increased Treg seen in CXCL10 KO mice. CXCL9, 10, and 11 are all Th1 chemoattractants and induced by IFN-γ. CXCL10 is also known as IP-10. It is produced by EC, VSMC, and macrophages. CXCL9 (Mig) and CXCL11 (I-TAC) are produced by EC and macrophages.</td>
<td>↓40%</td>
<td>CXCR3 (monocytes, Th1, mast cells)</td>
<td>CXCR3&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt; (1871). A clear reduction was seen early but no effect at the final time.</td>
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<tr>
<td>CXCL12 (SDF-1)</td>
<td>Pervascular siRNA CXCL12 in apoE&lt;sup&gt;−/−&lt;/sup&gt; mice after partial carotid ligation. CXCL12 attracts SMC progenitor cells to lesions with differentiation to lesional VSMC and increased fibrous cap thickness and reduced macrophage area (34). However, disease-associated human variant associated with higher CXCL12 expression (1186). Role in atherosclerosis remains controversial.</td>
<td>↑320%</td>
<td>CXCR4</td>
<td>CXCR4&lt;sup&gt;−/−&lt;/sup&gt; BMT apoE&lt;sup&gt;−/−&lt;/sup&gt; CXCL12/CXCR4 signaling mediates retention of neutrophils and VSMC progenitor cells in bone marrow. KO results in increase neutrophil count. Helped demonstrated that neutrophils are involved in atherosclerosis (2062).</td>
<td></td>
</tr>
<tr>
<td>CXCL13</td>
<td>VSMC are stimulated by LTβ to express CXCL13 and CCL21 which then direct lymphoid organogenesis in aortic adventitia (649).</td>
<td>—</td>
<td>CXCR5</td>
<td>CXCR5&lt;sup&gt;−/−&lt;/sup&gt; in C57BL/6 model of transplant arteriosclerosis (480). NS</td>
<td></td>
</tr>
<tr>
<td>CXCL16 (also on VSMC, monocytes)</td>
<td>CXCL16&lt;sup&gt;−/−&lt;/sup&gt; LDLR&lt;sup&gt;−/−&lt;/sup&gt; (83). CXCL16 also acts as a scavenger receptor on macrophages and this function may be atheroprotective. When cleaved by ADAM10 it can act as a chemoattractant.</td>
<td>↑57%</td>
<td>CXCR6 (Th1, NKT, VSMC)</td>
<td>CXCR6&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt; (579).</td>
<td>↓22%</td>
</tr>
<tr>
<td>CXCL19, 20</td>
<td>Antibodies to CXCL19 and CXCL20 decreased regression after transplanting atherosclerotic carotid arteries from apo E KO mice into wild-type mice (1810)</td>
<td>—</td>
<td>CXCR7</td>
<td>Induced when carotid arteries from apo E KO mice were transplanted into wild-type mice (1810). Also a receptor for CXCL12 but is not expressed on human or mouse leukocytes (127).</td>
<td></td>
</tr>
<tr>
<td>CX3CL1 (fractalkine—expressed primarily in VSMC rather than EC)</td>
<td>CX3CL1&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt; CX3CL1&lt;sup&gt;−/−&lt;/sup&gt; LDLR&lt;sup&gt;−/−&lt;/sup&gt; Reductions given for brachiocephalic artery (apoE&lt;sup&gt;−/−&lt;/sup&gt; NS at aortic root, while LDLR&lt;sup&gt;−/−&lt;/sup&gt; was significant at both sites.) (1777). Combined CX3CL1&lt;sup&gt;−/−&lt;/sup&gt; and CCL2&lt;sup&gt;−/−&lt;/sup&gt; in apoE&lt;sup&gt;−/−&lt;/sup&gt; mice showed additive reductions in atherosclerosis, even though CX3CL1 is thought to recruit less inflammatory monocytes (1525).</td>
<td>↓85% ↓50%</td>
<td>CX3CR1</td>
<td>CX3CR1&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt; (344). Adhesion to CX3CL1 thought to promote macrophage retention in artery wall. Exposure to oxLDL results in increased expression of CX3CR1 and decreased expression of CCR2, mediated by PPARγ, thereby promoting retention (111). CX3CR1&lt;sup&gt;−/−&lt;/sup&gt; also decreased DC in lesions in proportion to reduced atherosclerosis (1061).</td>
<td>↓50%</td>
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Table 6.—Continued

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<thead>
<tr>
<th>Endothelium</th>
<th>Model</th>
<th>%Δ A</th>
<th>Leukocyte</th>
<th>Model</th>
<th>%Δ A</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-CSF (also known as CSF1. Secreted by activated macrophages as well.)</td>
<td>M-CSF/−/− apoE/−/− (1648).</td>
<td>86%</td>
<td>CSF1R (monocytes)</td>
<td>anti-CSF1 mAb apoE/−/−</td>
<td>↓72%</td>
</tr>
<tr>
<td>VCAM-1, fibronectin</td>
<td>VCAM/−/− LDLR/−/− (VCAM/−/−, fibronectin See table 3 for fibronectin)</td>
<td>42%</td>
<td>α4β1 integrin (VLA4, CD49D/CD29)</td>
<td>α4 or β1 KO IS embryolethal. An α4 binding peptide (connecting segment 1 of fibronectin) was infused into LDLR/−/− mice to block VLA4 binding and signaling (1622). Decreased neointimal growth with VLA4 antibody (581). (Note: α4β7 also binds VCAM-1.)</td>
<td>↓66%</td>
</tr>
<tr>
<td>fibronectin</td>
<td>See table 3 for fibronectin EIIA domain defective in apo E KO mice (1751).</td>
<td>53%</td>
<td>αβ1 integrin</td>
<td>see above for β1</td>
<td>—</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>ICAM1/−/− LDLR/−/− (366). ICAM1/−/− in C57BL/6 (1272).</td>
<td>0%</td>
<td>α4β1 integrin (LFA1, CD11a/CD18)</td>
<td>ITGB2/−/− in C57BL/6. This effectively knocks out all 4 β2-containing integrins (1272). (Note: α4β1 also binds ICAM-1.)</td>
<td>↓45%</td>
</tr>
<tr>
<td>E-selectin</td>
<td>See above</td>
<td>30%</td>
<td>α4β1 integrin (CD11c/CD18)</td>
<td>ITGAM/−/− BMT into LDLR/−/− (957). MAC-1 also bound by GPIb/GPIX/GPV complex, possibly CD40L.</td>
<td>NS</td>
</tr>
<tr>
<td>ICAM-1, ICAM-2, fibronogen, collagen, iC3b, LPS</td>
<td>See above for ICAM-1</td>
<td>60%</td>
<td>α4β1 integrin (CD11c/CD18)</td>
<td>ITGAX/−/− (1986). CD11c can also be considered a marker for DC. Also see increase in CD11c positive monocytes in peripheral blood and greater activation markers of monocytes with high fat diet.</td>
<td>↓24%</td>
</tr>
<tr>
<td>vWF</td>
<td>See Table 10</td>
<td></td>
<td>GPibβ</td>
<td>GPibβ/−/− apoE/−/− (1689). Some GP (glycoprotein) receptors are not integrins while some are. GPib(α + β)/GPIX/GPV complex is a nonintegrrn receptor complex which signals through Src family activation and serves as the main vWF receptor on platelets.</td>
<td>NS</td>
</tr>
<tr>
<td>collagen, fibronectin</td>
<td>See above for fibronectin.</td>
<td>—</td>
<td>GPVI</td>
<td>GPVI, complexed with FcγR1, is the main platelet collagen receptor. Adenoviral over-expression of soluble or free GPVI (which inhibits normal GPVI binding) in carotid artery of cholesterol fed rabbits decreased atherosclerosis (as shown). Even greater effect was seen for anti-GPVI antibody in apoE KO mice. GPVI binding to fibronectin also shown (227).</td>
<td>↓38%</td>
</tr>
<tr>
<td>collagen</td>
<td>—</td>
<td>—</td>
<td>α4β1 integrin</td>
<td>ITGA1/−/− apoE/−/− (1550). Macrophage content of plaques was decreased and collagen and VSMC increased. Macrophages from α4−/− mice were unable to migrate on collagen IV. Similar phenotype to DDR1 deficiency (see Table 6)</td>
<td>↓42%</td>
</tr>
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Table 6.—Continued

<table>
<thead>
<tr>
<th>Endothelium</th>
<th>Model</th>
<th>%Δ A</th>
<th>Leukocyte</th>
<th>Model</th>
<th>%Δ A</th>
</tr>
</thead>
<tbody>
<tr>
<td>collagen</td>
<td>—</td>
<td>—</td>
<td>αβ integrin</td>
<td>ITGA2&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt; In platelets, αβ&lt;sub&gt;1&lt;/sub&gt; integrin acts together with GPVI to promote hemostasis but without effect when deleted solely (1678). Anti-inflammatory signaling in EC but no effect on atherosclerosis (659). NS</td>
<td></td>
</tr>
<tr>
<td>fibrinogen, vWF, fibronectin</td>
<td>See Table 10 for vWF. See above for fibronectin.</td>
<td>—</td>
<td>αβ integrin (platelets)</td>
<td>ITGA2&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt; (1150). Aortic lesion area given. Greater effect on carotid area (↓ 51%). ↓ 20%</td>
<td></td>
</tr>
<tr>
<td>vitronectin (VN), Thbs1</td>
<td>VN&lt;sup&gt;−/−&lt;/sup&gt; carotid stenosis</td>
<td>—</td>
<td>αβ integrin</td>
<td>This integrin also binds VCAM-1 —</td>
<td></td>
</tr>
<tr>
<td>Thbs1&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Increase intimal hyperplasia seen in one study of VN&lt;sup&gt;−/−&lt;/sup&gt; mice (398) but less in another (1422). Thbs1 (thrombospondin 1) and Thbs2 are type A Thbs which are endothelial-secreted extracellular matrix proteins. Initial more rapid progression then possible later benefit cancelled effect. Thbs1 deficient macrophages showed impaired phagocytosis (efferocytosis) and plaques showed more necrosis and elastic lamina degradation, and increased MMP9. Also binds CD36, and CD47. It may form a bridge between αβ&lt;sub&gt;3&lt;/sub&gt; integrin and CD36 to promote efferocytosis.</td>
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<tr>
<td>Thbs4&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt; (553)</td>
<td>A type B Thbs. Increases EC activation also neutrophil migration, activation (1423)</td>
<td>↓ 32%</td>
<td>αβ integrin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MADCAM1</td>
<td>(Also bound by L-selectin). Apparently no studies in atherosclerosis-prone mice.</td>
<td>—</td>
<td>αβ integrin</td>
<td>This integrin also binds VCAM-1 —</td>
<td></td>
</tr>
<tr>
<td>Transmigration</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>JAM-A</td>
<td>JAM-A&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt; Decreased neointimal formation after wire injury (2063).</td>
<td>—</td>
<td>LFA1, JAM-A</td>
<td>—</td>
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<tr>
<td>JAM-B</td>
<td></td>
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<tr>
<td>JAM-C ([also in VSMC])</td>
<td>Ab blocking in apoE&lt;sup&gt;−/−&lt;/sup&gt; mice led to decrease monocyte entry (1597).</td>
<td>—</td>
<td>MAC1, JAM-B</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>CD99</td>
<td>antibody to CD99 (1865).</td>
<td>↓ 38%</td>
<td>CD99</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>PECAM-1</td>
<td>see Table 3</td>
<td>see Table 3</td>
<td>PECAM-1</td>
<td>see Table 3</td>
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Ligands and receptors may be shared by several leukocytes and platelets, but monocyte interactions are generally emphasized. Several of the chemokine interactions are also involved in leukocyte/VSMC cross-talk in advanced lesions as indicated. Reference numbers are given in parentheses.

and Gα<sub>12/13</sub> (1604, 1950). Gα<sub>q</sub> when GTP bound, actively suppresses adenylate cyclase (AC) activity and thereby reduces cellular cAMP levels. While neither Gα<sub>q</sub> nor Gα<sub>12/13</sub> proteins directly inhibit AC, they do not promote cAMP formation and have downstream signaling effects that, in a number of instances, are directly opposite those of Gα<sub>q</sub>-coupled receptors and cAMP (1950). The βγsubunits released by these receptors have a number of effectors of their own including PI3Kγ and
PI3Kβ (followed by Akt/PKB activation and downstream signaling) and PLCβ (releasing IP₃ and DAG, followed by calcium release and activation of conventional PKCs). PLCβ is further activated indirectly by active Gα₁₁ binding while Gα₁₂/₁₃ directly activates several Rho GEFs (1950). PI3Kγ production of PIP₃ can lead to activation of downstream tyrosine kinases with phosphorylation of Shc followed by Grb2-SOS binding to Shc and then ERK1/2 activation. PYK2 activation by Gα₁₁ receptors has not been examined extensively in chemokine receptors, at least in relation to atherosclerosis. PYK2 increases endothelial permeability after leukocyte binding to endothelial ICAM-1 (54). Most chemokine receptors are found on the leading edge of migrating cells, and the PIP₃ formation helps provide polarization and directionality to the cell.

Newer insights disclose more diverse signaling capabilities of GPCR (150, 1372). Binding of several different adapter proteins to phosphorylated receptors couples GPCR to numerous pathways. Thus Src and JAK-STAT signaling can be activated downstream of β-arrestin binding to Gα₁coupled receptors. The adapter CNK1 (connector enhancer of ksr) links Gα₁₂/₁₃ receptors to simultaneous Rho activation and activation of the JNK cascade. Gα₆ can bind and activate Rho and Rac1 GEFs. Activation of MAPK together with intracellular calcium mobilization can activate cPLA₂ with release of arachidonic acid for eicosanoid synthesis.

The global effect of raising cAMP on expression of inflammation-related proteins was recently examined in an insightful study (1672). Human neutrophils were activated by a cocktail including LPS, formyl-methionyl-leucyl phenylalanine, GM-CSF, TNF-α and IL-1β. Then, either an A₂AR agonist, PGE₂, or a phosphodiesterase inhibitor were added separately to elevate cAMP. Results were remarkably similar for all three cAMP elevating agents. Transcription of several anti-inflammatory proteins was increased by cAMP elevation, including A20, COX-2, SOCS3, phosphatases DUSP1 and 2 (dual-specificity phosphatase 1 and 2, which can inactivate MAPK by dephosphorylating both tyrosine and serine residues), NR4A3 (nuclear receptor 4A3), and IER-2 (immediate early response 2, a GAP for Gα proteins). Downregulated proteins included pro-inflammatory transcription factors Egr2/3 as well as TNF-α, CCL3, and endothelin-1. While only neutrophils were examined, a variety of anti-inflammatory effects can be expected as a result of elevating cAMP in other cells as well. NR4A3 is a transcription factor whose expression is induced by NF-κB. NR4A3, in turn, can induce endothelial VCAM-1 and ICAM-1 expression but may also provide feedback inhibition in macrophages or other leukocytes (2096). Whole body KO of NR4A3 decreased atherosclerosis (175, 2096). Firm adhesion and diapedesis requires leukocyte integrin activation as shown in FIGURE 15. Briefly, GPCR binding by chemokines frees βγ subunits to activate PI3Kγ, PI3Kβ, and PLCγ followed by generation of DAG and IP₃ which releases ER calcium after binding IP₃R. Mobilized calcium can activate the sequence CalDAG-GEF1-Rap1-RIAM-talin-β integrin (66, 1605). This signaling occurs in <1 s. Integrin clustering and up-down polarization are established within 30 s, while spreading, lamellipodia formation, and crawling with formation of a clear leading and trailing edge occur in 30–300 s (1783). At the leading edge of the leukocyte, oriented toward the chemokine gradient, activated G proteins Rac1 and Cdc42 mediate lamellipodia and filopodia extension by serving to both orchestrate the machinery of actin microfiber polymerization, which drives membrane protrusion, and to anchor the growing chains to the cell membrane. Toward the rear, active RhoA mediates uropod retraction. Firmly adherent integrins anchor the cell to endothelial adhesion molecules in the middle. As the cell advances, integrins are detached toward the rear of the cell and recycled by cellular motors to the front.

Activated integrins on leukocytes bind VCAM-1 and ICAM-1 presented on activated endothelial cells. Macrophages express integrins that specifically bind fibronectin which is presented by endothelial integrins. Like the selectins, endothelial cell adhesion molecules should not be considered as passive Velcro as binding triggers endothelial cell intracellular signaling. For example, PYK2 activation, stimulated by leukocyte binding to endothelial ICAM-1, was shown to help mediate endothelial permeability and leukocyte transmigration (54).

TCR activation leads to integrin activation by a series of adapters and tyrosine kinases followed by PKCβ, protein kinase D (PKD), and Rap1 activation by Rap1 GEFs (231). Similarly to endothelial cell signaling reviewed previously, leukocyte integrin binding to various adhesion molecules, fibronectin, fibrinogen, or other activators result in activation of Src or Src family tyrosine kinases, together with FAK and PK which mediate outside-in signaling. Extended lamellipodia and filopodia seek an opportune site for penetration and diapedesis or transmigration of the leukocyte into the subendothelium. This may occur by passage of the leukocyte between endothelial cells utilizing interactions with various gap junction proteins (paracellular migration) or directly through thinned segments of endothelial cells (transcellular migration).

Leukocyte transmigration between endothelial cells requires leukocyte integrins and other receptors to interact in a complex fashion with endothelial gap junction proteins such as JAMs, PECAM-1, and other transmembrane proteins listed in TABLE 6, sometimes with binding to the same protein expressed on the leukocyte and endothelial cell (ho-
mophilic binding, such as PECAM-1 on one cell binding to PECAM-1 on an adjacent cell). Such binding occurs for PECAM-1, the JAMs, and CD99. In this process, endothelial cells essentially exchange endothelial-endothelial cell linkages for similar homophilic binding with migrating leukocytes. Actin-myosin contractions in the endothelial cells (controlled by RhoA signaling analogous to that in leukocytes) further facilitate the opening of endothelial junctions to allow the transmigration of leukocytes (1018).

VII. CYTOKINES AND INNATE VERSUS ADAPTIVE IMMUNITY IN ATHEROSCLEROSIS

A. General Features of Cytokine Signaling

While chemokines are often considered a subtype of cytokines, the following discussion focuses on cytokines other than chemokines. During the ongoing recruitment of macrophages and other leukocytes to the subendothelial space, numerous cytokines amplify and modulate the inflammatory response. In the context of T-cell activation, the T-cell receptor recognizes a specific antigen, which, when combined with costimulator activation leads to proinflammatory cytokine production. The expression of the costimulator receptors are often influenced by the cytokines present. Many cytokines promote leukocyte activation, differentiation, or proliferation while actively inhibiting apoptosis. Some have inhibitory effects. Cytokines may be secreted or presented on the cell surface. They may lead to autocrine, juxtacrine, and/or paracrine signaling. Cytokine classification schemes may be based on structure, post-receptor signaling, proinflammatory or anti-inflammatory effects, or physiological function. In one notable review, cytokines were designated as pro-atherosclerotic or anti-atherosclerotic (1766). Others operate in innate or adaptive immunity pathways or both (1). Results of numerous genetic interventions are reviewed in Table 7.

The general classes of cytokine receptors include TNFR superfamily receptors, JAK/STAT receptors, and receptors that utilize SMAD signaling. TNF-α, IL-1, IL-18, and LTα signal through the TNFR superfamily which was reviewed previously and summarized in Table 2. IL-17 receptors are analogous to TNF receptors, with activation of NF-κB, but use a different adapter, ACT1 (activator 1 of NF-κB) rather than MyD88 or TRADD to recruit TRAF6 (574). ACT1 also acts as an E3 ubiquitin ligase to add K63 ubiquitin chains to TRAF6. IL-17 receptors can also activate MAPK pathways independently of ACT1.

Interferons and interleukins other than IL-1, IL-17, and IL-18 signal through JAK/STAT pathways (1766). JAK/STAT receptors exemplify receptors that lack intrinsic tyrosine kinase activity but which utilize non-receptor tyrosine kinases for activation. Some receptor tyrosine kinases seem also to be able to activate JAK/STAT pathways. Typical JAK/STAT receptors are dimeric. Ligand binding activates bound JAK tyrosine kinases which phosphorylate tyrosines on the receptor’s cytoplasmic tail. Several signaling molecules can then bind to these phosphorytrosine sites (such as Grb2-Sos with downstream MAPK activation), but in particular there is binding of STAT transcription factors. JAK then phosphorylates the STAT followed by STAT dimerization and translocation of the active STAT dimers to the nucleus. Suppressors of cytokine signaling (SOCS) proteins are negative feedback inhibitors that can both block the catalytic domain of JAK and ubiquitinate the receptor for proteolytic degradation.

TGF-β receptors activate SMAD signaling, a particularly ancient pathway. The term SMAD is an amalgam of abbreviations for two homologous proteins, namely, MAD (mothers against decapentaplegic, a Drosophila signaling protein that directs correct formation of the 15 imaginal discs in the embryo that give rise to subsequent structures such as the abdomen, legs, wings, etc.) and Sma (short for “small,” with mutations resulting in short tail size and body length in the roundworm, C. elegans); others consider SMAD to signify “sons of mothers against decapentaplegic.” Active TGF-β is a dimer which binds type II (TβRII) and type I (TβRI) TGF-β receptor components, causing their oligomerization. The approximation of TβRII and TβRI allows the constitutively active serine/threonine kinase of the TβRII cytoplasmic tail to phosphorylate and thus activate TβRI. In conjunction with SARA (SMAD anchor of receptor activation), the activated cytoplasmic serine/threonine kinase of the TβRI then phosphorylates SMAD2 and SMAD3 which join with SMAD4, translocate to the nucleus, and there act in a complex fashion as transcription factors or co-activators (subject to additional controls and modifications by other signaling pathways). Bone morphogenetic protein (BMP) receptors utilize SMAD1/5/8 rather than SMAD2/3 but also interact with SMAD4. SMAD6/7 serve as inhibitors of several steps in the pathway while SMURF1/2 (SMAD ubiquitlation regulating factors 1 and 2) are SMAD-specific ubiquitin ligases that act to terminate SMAD signaling.

Intracellular signaling downstream of cytokine receptors is complex. Several aspects of TNF-α signaling were presented previously. Gene expression profiling identified 400 genes that were up- or downregulated at least twofold after exposure of endothelial cells to IL-1β, IFN-γ, and TNF-α (540). Expression of ~600–1000 genes significantly increased or decreased during the transition of monocytes to macrophages in response to cytokines (1000, 1963). Further details of the molecular biology of cytokine signaling in relation to atherosclerosis has been extensively studied and is the focus of several recent, excellent reviews (61, 580, 1766).
Table 7.  Initiation and perpetuation of inflammation through cytokine and immune cell signaling (91 genes tested)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Model</th>
<th>%Δ A</th>
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<tbody>
<tr>
<td>FcγRI/III (FCGR1/3)</td>
<td>γ−/− apoE−/−</td>
<td>↓55%</td>
<td>KO of γ chain affects both the FcγRI (CD64) and FcγRIII receptors. Each have ITAM domains in their cytoplasmic tail. Both are activating IgG receptors responsible for antibody-dependent phagocytosis and other signaling in macrophages and other leukocytes. FcγRI can act as a co-receptor with platelet GPVI. Intermediate reductions in atherosclerosis were seen in γ−/− apoE−/− mice (757). While opsonized LDL may be a target, these KO mice showed generalized reduction in inflammation and NF-κB signaling which is triggered through a CARD9-Bcl10/MAALT1-TRAF6 pathway (714). Also confirmed in mice receiving Fc γ chain deficient BMT (1298).</td>
</tr>
<tr>
<td>FcγRIII (FCGR3, CD16)</td>
<td>Fcgr3−/− LDLR−/−</td>
<td>↓30%</td>
<td>An increase in IL-10 as well as IFN-γ production by CD4+ cells was seen. Lipids were slightly higher in double KO mice despite reduced atherosclerosis (895).</td>
</tr>
<tr>
<td>FcγRIIb</td>
<td>FcγRIIib−/− apoE−/−</td>
<td>↑40%</td>
<td>FcγRIIib is an inhibitory IgG receptor with a cytoplasmic ITIM domain on monocyte/macrophages, DC, B-cells. Only male mice were affected. Shown is effect at 34 wk; earlier effects were stronger. Inflammatory cytokines in aorta were higher as were plasma IgG directed against modified LDL (1191) Only abdominal aorta area at 12 wk assessed. Increase in anti-oxLDL IgG also seen (2093).</td>
</tr>
<tr>
<td>IL-6</td>
<td>IL-6−/− apoE−/−</td>
<td>↓28%</td>
<td>IL-6 is induced by IL-1. Synthesized by macrophages, activated EC, VSMC, and mast cells in response to PAMPs, IL-1, and TNFα. In liver, IL-6 binding results in release of acute phase reactants including CRP and SAA. IL-6 signaling, with STAT3 activation, combined with TGFβ leads to Th17 differentiation which may be protective. Though IL-6 infusion appears to increase atherosclerosis, effects of KO on atherosclerosis are variable. Effects were NS for both models in one study (↓26%) (918), but atherosclerosis was significantly increased in apoE knockout by 85% (468) and 73% (1552) in other studies.</td>
</tr>
<tr>
<td>gp130 (IL6ST in humans)</td>
<td>liver-specific gp130−/− in apoE−/−</td>
<td>↓43%</td>
<td>IL-6 signal transducer (IL6ST) is the human homolog of gp130, a transmembrane subunit present in all IL-6 related receptors and also a component of other IL receptors. Marked reductions in serum SAA seen. Greater relative reduction of plaque earlier. Association of variants with human disease also reported (1085). IL-6 trans-signaling involves complex formation between IL-6 and soluble IL-6R in plasma followed by binding and signaling through gp130. Trans-signaling may be responsible for the most pro-inflammatory aspects of IL-6 signaling, including suppression of Treg differentiation. A soluble form of gp130 acts as a natural inhibitor of trans-signaling and decreases atherosclerosis when infused as a sgp130-Fc fusion protein. EC ICAM-1 and VCAM-1 were decreased with decreased macrophage infiltration (1589).</td>
</tr>
<tr>
<td>IL-12</td>
<td>IL-12−/− apoE−/−</td>
<td>↓52%</td>
<td>Produced largely by macrophages and DC in plaque and promotes Th1 differentiation and proliferation. Can upregulate OCR5 expression. Other studies confirm pro-atherosclerotic effects. Potentiates IFN-γ production. Shown is atherosclerosis reduction at 30 wk. Effect NS by 45 wk (918).</td>
</tr>
<tr>
<td>IL-18</td>
<td>IL-18−/− apoE−/−</td>
<td>↓35%</td>
<td>IL-18 induces both Th1 and Th17 cells to produce IFN-γ [together with IL-12] (471, 918). IL-18-deficient mice have much higher cholesterol than wild-type mice, and this may modulate the expression of atherosclerosis. Another group reported more atherosclerosis in IL-18 knockout, but this was only true in mice fed the high cholesterol diet (1400). Overexpression decreases collagen content and promotes rupture (1300).</td>
</tr>
<tr>
<td>IL-20</td>
<td>intramuscular plasmid cDNA injection into apoE−/−</td>
<td>↑22%</td>
<td>IL-20 clearly increased angiogenesis (see Table 6) and may promote T-cell recruitment to lesions (296).</td>
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Table 7.—Continued

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<th>Gene</th>
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<tr>
<td>MIF</td>
<td>MIF−/− LDLR−/−</td>
<td>↓ 78%</td>
<td>MIF (macrophage migration inhibitory factor) deficiency greatly inhibited progression of lesions, decreased VSMC, MMPs, and cathepsins (1373). MIF is a “noncognate” agonist of CD74/CD44, as well as CXC2 and CXCR4, recruiting both macrophages and T-cells. In addition to chemokine GPCR effects, ERK is activated through β-arrestin1 and Src signaling (135, 1995). Inhibition caused atherosclerosis regression in apo E KO mice (135). MIF potentiates actions of TNF-α and LPS and induces P-selection in endothelial cells (302). Increased expression in endothelial cells, VSMC, monocyte/macrophages by oxLDL. Endothelial expression increased by ANG II, CD40L, ROS, and, unexpectedly, corticosteroids (2061).</td>
</tr>
<tr>
<td>DLL4</td>
<td>anti-DLL4 antibody in LDLR−/−</td>
<td>↓ 21%</td>
<td>DLL4 (delta-like 4) is a NOTCH ligand that can be expressed on macrophages as well as other cell types, including endothelial cells. Inhibition by DLL4 antibody decreased several markers of macrophage activation, decreased adiposity, and improved insulin sensitivity (562).</td>
</tr>
<tr>
<td>OPN (SPP1)</td>
<td>OPN−/− apoE−/− with ANG II infusion</td>
<td>↓ 67%</td>
<td>OPN (osteopontin, also known as SPP1 for secreted phosphoprotein 1) is secreted by proliferating EC, VSMC, and especially active macrophages and Th1 cells. OPN is found in extracellular matrix and acts as a chemoattractant, a stimulus of phagocytosis, and an inflammatory adhesion molecule through adherence to αvβ3 and other integrins (803, 1547). A marked decrease in atherosclerosis was seen in ANG II-accelerated model which was attributed entirely to leukocyte effects by BMT (217). ANG II was shown to induce macrophage OPN expression. In mice without ANG II infusion and fed a regular chow diet there was no significant effect of OPN KO. OPN also induced by high glucose in diabetes. Other studies showed effects consistent with a proatherogenic effect of OPN (1547).</td>
</tr>
<tr>
<td>GDF-15</td>
<td>GDF-15−/− BMT into LDLR−/−</td>
<td>↓ 89%</td>
<td>GDF-15 (growth differentiation factor 15) is in the TGF-β superfamily produced largely by macrophages but also liver. 4-week data shown. Reduction in atherosclerosis was NS by 12 wk but a decrease in lesion macrophages seen (392).</td>
</tr>
<tr>
<td>CB2</td>
<td>CB2−/− BMT into LDLR−/−</td>
<td>↑ 13% (NS)</td>
<td>CB2 (Cannabinoid receptor 2) deficiency in bone marrow had mildly significant effect on early atherosclerosis but minimal effect on more advanced lesions as shown here (409). Prior studies had suggested mild anti-inflammatory effects for tetrahydrocannabinol.</td>
</tr>
<tr>
<td>Ntn1</td>
<td>Ntn1−/− BMT to LDLR−/−</td>
<td>↓ 45%</td>
<td>Ntn1 (netrin-1) is an axonal guidance cue during development. Macrophages activated by oxLDL synthesize and secrete Ntn1. Binding of Ntn1 to its receptor Unc5b inhibited macrophage migration (1854).</td>
</tr>
<tr>
<td>GRK5</td>
<td>GRK5−/− apoE−/−</td>
<td>↑ 50%</td>
<td>GRK5 has multiple activities. Major anti-atherosclerotic activities include phosphorylation with increased ubiquitination and degradation of CCR2 in macrophages. Also marks PDGF receptor β in VSMC and inhibits lysine phosphorylation activity of the CSF1R in macrophages (1387).</td>
</tr>
<tr>
<td>NFAT5</td>
<td>NFAT5−/− apoE−/− BMT into apoE−/− mice</td>
<td>↓ 86</td>
<td>NFAT5 can be activated by hypertonicity but its regulation in lesional macrophages is poorly understood. Haploinsufficient macrophages showed impaired migration. Given is percent reduction in en face aortic atherosclerosis (700).</td>
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**Sphingolipid and eicosanoid signaling in macrophages**

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<tr>
<th>Gene</th>
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<tbody>
<tr>
<td>S1PR2</td>
<td>S1PR2−/− apoE−/−</td>
<td>↓ 82%</td>
<td>Sphingosine-1-phosphate acting through S1P receptors 1-5 (S1PR1-5, also known as S1P1-5) can be pro- or anti-inflammatory depending on the receptor. S1PR2 activates Rho and promotes endothelial permeability but also stimulates macrophages to produce IL-1β and IL-1β. Bone marrow transplant with S1PR2-deficient cells resulted in 65% reduction in lesions suggesting most of the reduction in atherosclerosis was from macrophage effects (1640).</td>
</tr>
<tr>
<td>S1PR3</td>
<td>S1PR3−/− apoE−/−</td>
<td>NS</td>
<td>Lesions had many fewer macrophages but increased VSMC. S1PR3-null macrophages showed less chemotaxis and less inflammatory activation while VSMC showed greater migration and proliferation (898).</td>
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Table 7.—Continued

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<th>Gene</th>
<th>Gene Model</th>
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<tr>
<td>12/15-LO</td>
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<tr>
<td>12/15-LO</td>
<td>12/15-LO−/− apoE−/−</td>
<td>† 63%</td>
<td>Assessed at 15 wk (aortic sinus) (368)</td>
</tr>
<tr>
<td></td>
<td>12/15-LO−/− LDLR+/+</td>
<td>† 77%</td>
<td>Assessed at 9 wk (aortic sinus) (605)</td>
</tr>
<tr>
<td></td>
<td>12/15-LO−/− BMT into apoE−/−</td>
<td>† 56%</td>
<td>Assessed at 12 wk (aortic surface area). All atherosclerosis effect was due to bone marrow cells (807).</td>
</tr>
<tr>
<td></td>
<td>human 15-LO Tg in WHHL rabbit</td>
<td>† 45%</td>
<td>WHHL rabbits were fed a high-fat, high-cholesterol diet and assessed at 13.5 wk. Enzyme was expressed essentially only in monocyte-macrophages (1813)</td>
</tr>
<tr>
<td></td>
<td>macrophage-specific 12/15-LO Tg in apoE−/− mice</td>
<td>† 31%</td>
<td>In chow-fed mice, overexpression of human 12/15-LO in macrophages increased production of LX4 (sixfold), protectins (PD1), and resolvins (RvD1) thought to help resolve inflammation. RvD1 and PD1 also showed strong anti-inflammatory effects in cultured endothelial cells (1197).</td>
</tr>
<tr>
<td></td>
<td>12/15-LO−/− BMT into apoE−/−</td>
<td>† 70%</td>
<td>BMT of 12/15-LO null cells increased atherosclerosis in chow-fed mice (1197).</td>
</tr>
<tr>
<td></td>
<td>macrophage-specific 12/15-LO Tg in apoE−/− mice</td>
<td>NS</td>
<td>Animals fed high fat Western diet in contrast to chow feeding in prior studies by this group (1198)</td>
</tr>
<tr>
<td></td>
<td>12/15-LO−/− BMT into LDLR+/+ mice fed high PUF or SAT</td>
<td>† 62%</td>
<td>In mice fed a high-PUF diet, BMT of 12/15-LO null cells clearly decreased atherosclerosis (1507). Whole body KD of 12/15-LO was also clearly protective in mice fed either high PUF or high SAT.</td>
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### Table 7.—Continued

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<tr>
<td><strong>Costimulatory and coinhibitory receptor-ligand pairs</strong></td>
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<tr>
<td>B7-1/2</td>
<td>B7-1/2&lt;sup&gt;−/−&lt;/sup&gt; LDLR&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↓ 37%</td>
<td>Whole body KO of both B7-1 (CD80) and B7-2 (CD86). B7-1/2 are constitutively expressed on antigen presenting cells and were found on plaque macrophages. Their common ligands are CD28 (activating) and CTLA4 (inhibitory) on T cells. Decrease in atherosclerosis shown was for intimal area at 20 wk but surface area was NS. Impaired IFNγ production by triple KO mice suggested mainly Th1 cell reduction (230).</td>
</tr>
<tr>
<td>B7-1/2&lt;sup&gt;−/−&lt;/sup&gt; BMT into LDLR&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑ 133%</td>
<td>Opposite results seen with BMT (33). In this model, mostly Treg cells appeared to affected (547).</td>
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<tr>
<td>CD28</td>
<td>CD28&lt;sup&gt;−/−&lt;/sup&gt; LDLR&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑ 107%</td>
<td>Again, Treg proliferation seemed to be most impaired by CD28 deficiency in BMT. Tregs transfer reversed the increase in atherosclerosis and markedly decreased Th cells in plaques (33).</td>
</tr>
<tr>
<td>ICOS</td>
<td>ICOS&lt;sup&gt;−/−&lt;/sup&gt; LDLR&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑ 43%</td>
<td>ICOS (inducible co-stimulator), a positive co-stimulator receptor on differentiating T-cells that binds ICOSL on antigen presenting cells. Promotes Treg proliferation especially (less so Th cells). ICOS-deficient mice had fewer Treg cells and increased Th cells with greater IFNγ and TNF-α production (646). No genetic atherosclerosis model reported for ICOSL.</td>
</tr>
<tr>
<td>PD-1</td>
<td>PD-1&lt;sup&gt;−/−&lt;/sup&gt; LDLR&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑ 73%</td>
<td>PD-1 (programmed death 1 receptor) is the receptor for PD-L1/2. Both CD4&lt;sup&gt;+&lt;/sup&gt; Th and CD4&lt;sup&gt;+&lt;/sup&gt; CTL cells were more activated in PD-1 KO mice (221).</td>
</tr>
<tr>
<td>PD-L1/2</td>
<td>PD-L1/2&lt;sup&gt;−/−&lt;/sup&gt; LDLR&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑ 90%</td>
<td>PD-L1 (programmed death ligand 1) and PD-L2 are coinhibitory ligands to PD-1 expressed on antigen presenting cells and Treg that bind their receptor PD-L1 expressed on activate Th cells. Aortic root at 19 wk shown. KO mice had many more active T-cells and macrophages lesions and serum TNF-α (645).</td>
</tr>
<tr>
<td>CD40</td>
<td>CD40&lt;sup&gt;−/−&lt;/sup&gt; LDLR&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>NS</td>
<td>On EC, VSMC, T-cells, B-cells, DC, and macrophages. CD40L interaction with Mac1 may explain the lack of effect with CD40&lt;sup&gt;−/−&lt;/sup&gt;. Treatment of LDLR&lt;sup&gt;−/−&lt;/sup&gt; mice with anti-Mac1 antibodies resulted in a 50% reduction in lesion size with fewer macrophages (2119).</td>
</tr>
<tr>
<td>CD40&lt;sup&gt;−/−&lt;/sup&gt; ApoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↓ 42%</td>
<td>Reduction in atherosclerosis quite variable (not significant in thoracic aorta, only abdominal). Plaques had fewer macrophages and T-cells and more collagen (1093).</td>
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<tr>
<td>CD40&lt;sup&gt;−/−&lt;/sup&gt; BMT into LDLR&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↓ 45%</td>
<td>Slightly more significant reduction in atherosclerosis than whole body CD40 knockout (1093).</td>
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<tr>
<td>CD40L (CD154)</td>
<td>CD40L&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↓ 82%</td>
<td>Mostly on activated T-cells and platelets but also DC, macrophages, EC, VSMC. Absence of CD40L had the greatest effect on advanced lesions and resulted in a more stable plaque phenotype (1092).</td>
</tr>
<tr>
<td>CD137 (4-1BB)</td>
<td>CD137&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↓ 39%</td>
<td>Expressed on activated EC, CD4&lt;sup&gt;+&lt;/sup&gt; and CD8&lt;sup&gt;+&lt;/sup&gt; T cells, resting monocytes, and DC. Expression increased by TNF-α. CD137 ligation by CD137L on stimulated monocytes/macrophages, DC, activated B cells stimulates IFN-γ production by T cells and VCAM-1, ICAM-1, MCP-1, and IL-6 by EC (1348). Monocyte adhesion decreased to CD137&lt;sup&gt;−/−&lt;/sup&gt; and EC and aortic endothelium. BMT studies showed most of the reduction in atherosclerosis in CD137 deficient mice was due to bone marrow-derived cells. Macrophage expression of CD137 may be induced by oxLDL (847).</td>
</tr>
<tr>
<td>CD137&lt;sup&gt;−/−&lt;/sup&gt; LDLR&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↓ 31%</td>
<td>Costimulatory or bidirectional signaling shown by incubating CD137L-expressing macrophages with cell-free CD137 protein (CD137-Fc) which led to marked increase in macrophage production of TNF-α and MCP-1. CD137&lt;sup&gt;−/−&lt;/sup&gt; mice had lower levels of TNF-α and MCP-1 in lesions (847). No genetic atherosclerosis model reported for CD137L.</td>
<td></td>
</tr>
<tr>
<td>OX40L (TNFSF4)</td>
<td>OX40L&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↓ 59%</td>
<td>Considered underlying gene of Ath-1 locus (1917). OX40L is expressed on activated EC, B-cells, and macrophages. OX40L KO suppressed VEGF expression and vaso vasorum neovascularization. OX40L&lt;sup&gt;−/−&lt;/sup&gt; BMT had no effect (1278). Similar results with anti-OX40L antibody treatment (1864). No genetic atherosclerosis model reported for OX40L (also known as CD134).</td>
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**Related to neutrophils**

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<tbody>
<tr>
<td>IRF8</td>
<td>IRF8−/− BMT into apoE−/−</td>
<td>↑ 142%</td>
<td>IRF8 [interferon regulatory factor] deficiency in mice leads to a chronic myelogenous leukemia-like phenotype with increased neutrophils in the circulation. Larger necrotic core seen. Similar increase with IRF8 deficient BMT into LDLR KO mice. Macrophages had impaired efferocytosis capacity and secreted less IL-10 but atherosclerosis normalized with depletion of neutrophils (440).</td>
</tr>
<tr>
<td>MPO</td>
<td>Human MPO Tg BMT into LDLR−/−</td>
<td>↑ 136%</td>
<td>MPO [myeloperoxidase] is not expressed in mouse macrophages or neutrophils. Interestingly, transplant of MPO KO cells resulted also in increased atherosclerosis in this study (1176).</td>
</tr>
<tr>
<td>MPO−/− BMT into LDLR−/−</td>
<td>↑ 52%</td>
<td>The low MPO activity seemed to be associated with protective signaling in this and the above study (207).</td>
<td></td>
</tr>
<tr>
<td>CRAMP</td>
<td>CRAMP−/− BMT into LDLR−/−</td>
<td>↓ 52%</td>
<td>CRAMP [cathelicidin related antimicrobial peptide], released by neutrophils in extracellular nets complexes with self-DNA to activate plasmacytoid dendritic cells through TLR9 (439).</td>
</tr>
<tr>
<td>FAAH</td>
<td>FAAH−/− apoE−/−</td>
<td>↓ 21%</td>
<td>FAAH [fatty acid amide hydrolase] catabolizes the endocannabinoid anandamide, a fatty acid amide. Though plaques were smaller, there was an increase in neutrophil content and decreased VSMC, suggesting a more vulnerable phenotype. Increased neutrophil recruitment appeared to be due to local increased production of the neutrophil chemokine CXCL1. Additionally, fewer Treg were found in spleens of FAAH-deficient mice. Findings were thought to be consistent with decreased atherosclerosis by rimonabant treatment in LDLR-null mice (1005).</td>
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**Related to NK or NKT [natural killer or natural killer T-cells]**

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<tbody>
<tr>
<td>Ly49A</td>
<td>Ly49A Tg (overexpressing) BMT into LDLR−/−</td>
<td>↓ 70%</td>
<td>Ly49A is a NK receptor that inhibits NK activation when bound to class I MHC. There was a marked decrease in number as well as NK activity in Ly49A Tg transplanted mice. This model was thought to an improvement over the beige mouse which also lacks NK cells but has other phenotypes (1051, 1955).</td>
</tr>
<tr>
<td>KLRK1</td>
<td>KLRK1−/− apoE−/−</td>
<td>↓ 80%</td>
<td>KLRK1 [killer cell lectin-like receptor, subfamily K, member 1; also known as NKG2D for NK cell lectin-like receptor subfamily K] is a membrane-bound receptor expressed on NK cells, NKT cells, γδ T cells, and CD8+ αβ T cells. Ligands are proteins expressed on damaged or virus-infected cells. IL-6, IL-12, and IFN-γ were reduced in KLRK1 KO mice (1989).</td>
</tr>
<tr>
<td>CD1d1</td>
<td>CD1d1−/− apoE−/−</td>
<td>↓ 67%</td>
<td>CD1 receptors present lipid antigens to various T cells. CD1d1 is recognized by NKT cells. Injection of α-galactosylceramide, a synthetic glycolipid, nearly doubled lesions in apoE−/− but not in CD1d1−/− apoE−/− (1119). Similar but less marked changes reported by another group (1821).</td>
</tr>
<tr>
<td>CD1d1−/− LDLR−/−</td>
<td>↓ 47%</td>
<td>Reduction in atherosclerosis seen only at 4 wk and NS at 8 and 12 wk (B2)</td>
<td></td>
</tr>
<tr>
<td>Jα18</td>
<td>Jα18−/− LDLR−/−</td>
<td>↓ 24%</td>
<td>A subset of NKT have an invariant T-cell receptor [TCR] with the α-chain composed of Vα14 joined with Jα18. This α chain can join with any one of 3 β chains. This invariant TCR binds CD1d. Less IFN-γ, was seen in lesions as well (1498). Invariant NKT express less Ly49A and are the major contributors of increased atherosclerosis as compared to other (CD4-CD8-) NKT (1797).</td>
</tr>
</tbody>
</table>

**Secreted by or related to mast cells**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Model</th>
<th>%Δ A</th>
<th>Function, Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDC</td>
<td>HDC−/− apoE−/−</td>
<td>↓ 40%</td>
<td>HDC [histidine decarboxylase] is the rate-limiting enzyme for histamine synthesis (1900).</td>
</tr>
<tr>
<td>HRH1</td>
<td>HRH1−/− apoE−/−</td>
<td>↓ 80%</td>
<td>HRH1 [histamine receptor H1] deficient mice had decreased vascular permeability to LDL. Transplant of HRH1−/− bone marrow did not affect atherosclerosis (1513).</td>
</tr>
<tr>
<td>HRH2</td>
<td>HRH2−/− apoE−/−</td>
<td>NS</td>
<td>No effect on atherosclerosis with H2 antagonist (ranitidine) as well (1515).</td>
</tr>
<tr>
<td>Kit</td>
<td>KitNsh/Wsh LDLR−/−</td>
<td>↓ 83%</td>
<td>Kit is a transmembrane tyrosine kinase receptor for stem cell factor (SCF), a growth factor required for mast cell differentiation (1709).</td>
</tr>
</tbody>
</table>

Continued
### Table 7—Continued

<table>
<thead>
<tr>
<th>Gene</th>
<th>Model</th>
<th>%Δ A</th>
<th>Function, Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM-CSF</td>
<td>GM-CSF−/− LDLR−/−</td>
<td>↓ 35%</td>
<td>Average of LDLR−/− studies shown (918). Injection of GM-CSF promoted proliferation of intimal cells, particularly dendritic cells in LDLR−/− mice (2112).</td>
</tr>
<tr>
<td></td>
<td>GM-CSF−/− apoE−/−</td>
<td>↑ 100%</td>
<td></td>
</tr>
<tr>
<td>Rag1</td>
<td>Rag1−/− apoE−/− Chow fed</td>
<td>↓ 42%</td>
<td>Only chow fed (with lower serum cholesterol) showed a significant effect on lesion size (379). In another study, early (8-week) atherosclerosis was reduced in Rag1−/− apoE−/− mice (54% reduction) but differences diminished to NS with time (1653). Estrogen-deficient female mice showed 50% or greater reduction of atherosclerosis, but estrogen-replete mice had reduced lesions with no effect of Rag1−/− (470).</td>
</tr>
<tr>
<td></td>
<td>Rag1−/− apoE−/− Western diet</td>
<td>↓ 12%</td>
<td></td>
</tr>
<tr>
<td>Rag2</td>
<td>Rag2−/− apoE−/− 27 wk</td>
<td>↓ 81%</td>
<td>Of note, the Rag2 deficient mice had considerably lower plasma triglyceride levels. There was no difference in brachiocephalic lesions (1468). In another study, lesions in Rag2−/− and Rag2−/− mice were not significantly different. Despite lack of B and T-cell derived cytokines (including IFN-γ and IL-4 which stimulate expression of MHC III on macrophages), foam cell formation was not affected (382).</td>
</tr>
<tr>
<td></td>
<td>Rag2−/− apoE−/− 40 wk</td>
<td>↓ 57%</td>
<td></td>
</tr>
<tr>
<td>PRKDC</td>
<td>scid/scid apoE−/−</td>
<td>↓ 66%</td>
<td>A spontaneous mutation in PRKDC (protein kinase, DNA-activated, catalytic subunit) causes the scid/scid (recessive severe combined immunodeficiency) trait in mice. The gene allows antigen receptor diversification by V(D)J recombination by opening blunt-end hairpins created by Rag1/Rag2. The mice have virtually no T or B cells (76). The marked reduction in atherosclerosis was largely reversed by T-helper cell transfer (2107).</td>
</tr>
<tr>
<td>CD4</td>
<td>CD4−/− apoE−/−</td>
<td>↓ 70%</td>
<td>CD4 is a TCR co-receptor or adapter which defines the Th (CD4+) lineage. CD4 KO mice had no Th cells and much less IFN-γ in lesions (2108). Another group found no effect (469, 470).</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>TCRβ</td>
<td>TCRβ−/− apoE−/−</td>
<td>↓ 49%</td>
<td>In female mice fed chow diet. TCRβ is a subunit of the TCR found in CD4+ and CD8+ cells (469). Confirmed in a separate study (470).</td>
</tr>
<tr>
<td>TCRδ</td>
<td>TCRδ−/− apoE−/−</td>
<td>↓ 49%</td>
<td>In female mice fed chow diet. TCRδ is a subunit on a small subset of T cells (469). Confirmed in a separate study (470).</td>
</tr>
<tr>
<td>Related to <strong>Th1</strong> cells. Listed cytokines are increased after activation, typically by antigen presentation (DC or macrophage), reinforced by CD40-CD40L costimulation.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>STAT4</td>
<td>STAT4−/− in BALB/c fed Paigen diet</td>
<td>NS</td>
<td>Like the control BALB/c mice, the STAT4 KO in this study had no atherosclerosis. STAT4 KO in this study had no atherosclerosis. STAT4 is required for development of more atherogenic Th1 and STAT4 KO resulted in a greater Th2 response (798).</td>
</tr>
<tr>
<td>IFNγ (IFNG)</td>
<td>IFNγ−/− apoE−/−</td>
<td>↓ 60%</td>
<td>Secreted primarily by Th1 but also natural killer T-cells (NKT), mast cells and activated macrophages. Activates endothelial cells, increases macrophage production of TNF-α and MCP-1 (918).LTα</td>
</tr>
<tr>
<td></td>
<td>IFNγ−/− LDLR−/−</td>
<td>↓ 43%</td>
<td></td>
</tr>
<tr>
<td>IFNγR1</td>
<td>IFNγR1−/− apoE−/−</td>
<td>↓ 58%</td>
<td>The interferon-γ receptor is a heterodimer of the high-affinity IFNγR1 subunit and the accessory IFNγR2 subunit. Presumably, IFNγR1 was deleted in this study. Plaques had more fibrous tissue, fewer inflammatory cells (678).</td>
</tr>
<tr>
<td>IL-2</td>
<td>IL-2 mAb apoE−/−</td>
<td>↓ 20%</td>
<td>In cited study, monoclonal antibody (mAb) was administered intraperitoneally (1835).</td>
</tr>
<tr>
<td>Related to <strong>Th2</strong> cells.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-4</td>
<td>IL-4−/− apoE−/−</td>
<td>↓ 27%</td>
<td>Produced by Th2 lymphocytes and mast cells. Required for Th2 differentiation and M2 macrophage phenotype. Can be pro- or anti-inflammatory with mixed effects on atherosclerosis generally (913, 918, 1766).</td>
</tr>
<tr>
<td></td>
<td></td>
<td>or NS</td>
<td></td>
</tr>
<tr>
<td>IL-5</td>
<td>IL-5−/− BMT into LDLR−/−</td>
<td>↑ 50%</td>
<td>Produced by Th2 lymphocytes and mast cells. Appears to be anti-atherosclerotic. IL-5 stimulates innate B1-cells to produce an IgM natural antibody against oxidized phospholipid (147). Also promotes eosinophil differentiation.</td>
</tr>
</tbody>
</table>

*Continued*
IL-10 IL-10
IL-13 IL-13
CYP24 CYP24
Tg rats on high
fat diet

TGF-

CD25 anti-CD25 antibody in

ST2 Infusion of sST2 (decoy
receptor) in apoE

STAT6 STAT6

IL-17R IL-17R

Related to Th17 cells

IL-17A IL-17A

IL-17A in CS7BL/6
fed high fat diet and
infected with C.

IL-17A apoE

IL-17R IL-17R

SOCS3 SOCS3

SOCS3 (suppressor of cytokine signaling 3) deficiency in T-cells led to

Related primarily to T-regulatory cells (Treg)

CD3 anti-CD3 antibody in

CD25 anti-CD25 antibody in

Foxp3 vaccination with activated
DCs overexpressing
Foxp3 in LDLR

TGF-β1 TGF-β1 conditional Tg
expressed in heart
controlled by
doxycycline in apoE

TGF-β receptor
II (TGFBR2) dominant negative TGF
receptor II in
apoE

IL-10 IL-10

IL-10 in BMT into

CYP24 CYP24

Table 7.—Continued

<table>
<thead>
<tr>
<th>Gene</th>
<th>Model</th>
<th>%Δ A</th>
<th>Function, Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-13</td>
<td>IL-13&lt;sup&gt;-/-&lt;/sup&gt; BMT into LDLR&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>↑91%</td>
<td>Administration of IL-13 decreased macrophage content, markers of inflammation, and increased collagen content of established lesions while deficiency led to increased plaque size (258).</td>
</tr>
<tr>
<td>IL-33</td>
<td>IL-33 infusion in apoE&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>↓62%</td>
<td>Increased atherosclerosis seen in mice infused with a soluble receptor for IL-33 which blocked IL-33 signaling (1209). Stimulates IL-5 secretion (1130).</td>
</tr>
<tr>
<td>ST2</td>
<td>Infusion of sST2 (decoy receptor) in apoE&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>↑89%</td>
<td>ST2 (suppressor of tumorigenicity 2) is the receptor for IL-33. ST2L is the active, membrane-bound receptor, while sST2 is a soluble decoy receptor which binds IL-33 and suppresses ST2L signaling (1209).</td>
</tr>
<tr>
<td>STAT6</td>
<td>STAT6&lt;sup&gt;-/-&lt;/sup&gt; in BALB/c fed Paigen diet</td>
<td>↑&gt;400%</td>
<td>The control BALB/c mice display a predominantly Th2 immune response and had no detectable atherosclerosis despite the Paigen diet. STAT6 is required for Th2 differentiation (798).</td>
</tr>
<tr>
<td>IL-17A</td>
<td>IL-17A&lt;sup&gt;-/-&lt;/sup&gt; in CS7BL/6 fed high fat diet and infected with C. pneumoniae</td>
<td>↓50%</td>
<td>IL-17A (the first identified of 6 IL-17 family members) secreted by newly designated Th17 activates endothelial cells and macrophages (293). Prior antibody or transgenic expression studies controversial (1748).</td>
</tr>
<tr>
<td>IL-17A apoE</td>
<td>IL-17A&lt;sup&gt;-/-&lt;/sup&gt; apoE&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>↑62%</td>
<td>In apo E null mice, plaques in IL-17A deficient animals had more macrophages, fewer VSMC, less collagen. Splenic CD4&lt;sup&gt;+&lt;/sup&gt; cells had more IFNγ early on and less IL-5 throughout lesion development. Exogenous IL-17A administration decreased atherosclerosis [380].</td>
</tr>
<tr>
<td>IL-17R</td>
<td>IL-17R&lt;sup&gt;-/-&lt;/sup&gt; BMT into LDLR&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>↓46%</td>
<td>The IL-17 receptor (IL-17R) is found in multiple bone-marrow derived cell types as well as endothelial cells. Mice transplanted with IL-17R KO cells had decreased IL-6 and increased IL-10 production (1853).</td>
</tr>
<tr>
<td>SOCS3</td>
<td>T-cell-specific SOCS3&lt;sup&gt;-/-&lt;/sup&gt; (Cre-Lox) BMT into LDLR&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>↓50%</td>
<td>SOCS3 (suppressor of cytokine signaling 3) deficiency in T-cells led to surprising decrease in atherosclerosis, increased IL-10 and IL-17 but decreased IFNγ production and induced an anti-inflammatory macrophage phenotype. Transplant of T-cells overexpressing SOCS3 into Rag2&lt;sup&gt;2&lt;sup&gt;/-&lt;/sup&gt;, ApoE&lt;sup&gt;-/-&lt;/sup&gt; mice greatly increased atherosclerosis. IL-17 possibly protective in some settings (1747).</td>
</tr>
<tr>
<td>CD3</td>
<td>anti-CD3 antibody in LDLR&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>↓59%</td>
<td>CD3 is an ITAM-containing co-receptor of the TCR complex. Shown are results when given iv for 5 days before starting high fat diet. When given after 13 wk of diet, there was 71% less progression over 11 wk. The antibody used was engineered to be missing the Fc portion and so was not pro-inflammatory (1677). Similar antibodies can stimulate TCR generally but cause a net increase in Treg number and suppress several autoimmune conditions.</td>
</tr>
<tr>
<td>CD25</td>
<td>anti-CD25 antibody in apoE&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>↑50%</td>
<td>Depletes Treg but not a definitive proof (33).</td>
</tr>
<tr>
<td>Foxp3</td>
<td>vaccination with activated DCs overexpressing Foxp3 in LDLR&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>↑34%</td>
<td>The vaccination reduced Treg cells specifically by about 30% in various tissues by sensitizing CTL with 1.917 fold increase in splenic T-cells (1852)</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>TGF-β1 conditional Tg expressed in heart controlled by doxycycline in apoE&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>↓20%</td>
<td>Shown here is aortic root atherosclerosis in animals overexpressing TGF-β1 versus doxycycline-suppressed controls (555). In another study, heterozygous TGF-β1 deficiency increased atherosclerosis in fat-fed C57BL/6 mice (551).</td>
</tr>
<tr>
<td>TGF-β receptor II (TGFBR2)</td>
<td>dominant negative TGF receptor II in apoE&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>↑200%</td>
<td>This model resulted in disruption of TGF-β signaling in T-cells only (1494). TGF-β supports Treg differentiation by acting as a costimulatory factor for Foxp3. Expression of a dysfunctional TGFBR2 only in CD11&lt;sup&gt;+&lt;/sup&gt; DC also increased atherosclerosis (1043).</td>
</tr>
<tr>
<td>IL-10</td>
<td>IL-10&lt;sup&gt;-/-&lt;/sup&gt; BMT into LDLR&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>↑35%</td>
<td>Also produced by Th2, alternatively activated (M2) macrophages, CD8&lt;sup&gt;+&lt;/sup&gt; cells, and B-cells. Anti-inflammatory. Promotes increase in anti-inflammatory Th2 cells relative to Th1 and tolerance. Several studies show anti-atherosclerotic effects (B18).</td>
</tr>
<tr>
<td>CYP24</td>
<td>CYP24 Tg rats on high fat diet</td>
<td>↑4336%</td>
<td>CYP24 is vitamin D&lt;sub&gt;2&lt;/sub&gt; hydroxylase. 24-hydroxilation by CYP24 of 1,25 dihydroxy D&lt;sub&gt;2&lt;/sub&gt; leads to inactivation. These transgenic rats constitutively overexpress CYP24, had mild hyperlipidemia, and developed albuminuria. Normal rats even on high-fat diet had very little atherosclerosis (B86). A CYP24A1 variant was associated with coronary calcium but directional effect on vitamin D is not yet known (1611).</td>
</tr>
</tbody>
</table>
MHCI duplicated MHCI locus (3N in apoE−/−) ↓ 73% The added MHCI locus (1.9 Mb duplicated) included a number of other genes, complicating interpretation. 4N duplication was embryolethal. Potential mechanisms for effect were not explored (487).

CD8 CD8−/− apoE−/− NS CD8 is a TCR co-receptor or adapter which defines the cytotoxic T lymphocyte (CTL CD8+) lineage. In female mice fed chow diet in 2 studies by the same group (469, 470). Others report a significant reduction in atherosclerosis with depletion of CD8 by antibodies (972).

TAP1 TAP1−/− apoE−/− NS TAP1 (transporter associated with antigen processing 1) had no effect on atherosclerosis (932). TAP1 transports cytosol-derived antigens into the ER to be loaded onto MHCI which presents antigen to the TCR with the CD8 adapter (expressed on CTL).

PRF1 PRF1−/− CTL BMT into lymphocyte-deficient apoE−/− ↓ 34% PRF1 (perforin, also known as PFP for pore forming protein) replete (wild-type) CTL increased atherosclerosis when transplanted into lymphocyte-deficient animals. Shown is the difference between these two models (972).

GZMB GZMB−/− CTL BMT into lymphocyte-deficient apoE−/− ↓ 44% GZMB (granzyme B)-deficient CTL were compared with wild-type CTL transfer as in the PRF1 study above (972).

B-cell receptor

IgM IgM-transmembrane defective BMT into LDLR−/− ↑ 29% B-cell receptor function of IgM depends on normal transmembrane sequence. Mice with disrupted exons encoding the transmembrane sequence (so called µMT mice) had complete absence of B-cells and increased atherosclerosis (1117). Nonsignificant effect in female ApoE-deficient mice fed high-fat diet. There was a 6.1-fold increased on chow diet. Lesions showed more apoptosis and cholesterol crystals. Simultaneous deletion of C1q did not make lesions worse (1015).

Secreted IgM Secreter IgM-defective LDLR−/− ↑ 29% B-cells from secretory IgM-defective mice could not secrete natural IgM antibodies derived from B1a cells. Given here are data from high-fat diet. There was a 6.1-fold increased on chow diet. Lesions showed more apoptosis and cholesterol crystals. Simultaneous deletion of C1q did not make lesions worse (1015). Adoptive transfer study. Splenectomy decreased B1a cells and increased plaques. Adoptive transfer of wild-type B1a cells (used as comparator here) was strongly anti-atherosclerotic, but not transfer of secretory IgM-deficient B1a cells (970).

Id3 Id3−/− BMT into apoE−/− ↑ 210% Id3 (inhibitor of DNA binding 3) is an inhibitory transcription factor that appears to be required for normal homing of B-cells to the aortic adventitia, possibly through (indirect?) upregulation of CCR6 and other effects (437). Protective effect was not mediated by IgM, but rather a reduction in macrophage accumulation, suggesting possible release of a suppressive chemokine or cytokine such as a Breg cell function.

BAFFR BAFFR−/− apoE−/− ↓ 44% BAFF (B cell activating factor belonging to the TNF family) and BAFFR, its receptor, are required for B2 cell development. BAFFR−/− animals were deficient in B2 cells but had normal B1a cell numbers with a decrease in IgG1 and IgG2c antibodies in plasma and lesions but much lesser effect on IgM. VCAM-1 expression was decreased as well (968). Similar results for LDLR null animals with BMT with decreased IgG against malondialdehyde-modified LDL (1528).

BAFFR−/− BMT into LDLR−/− ↓ 33%

BAFFR−/− BMT into LDLR−/− ↓ 33%

Related to dendritic cells (DC). Can be defined as CD11c+c, MHCIhi and includes conventional DC derived from monocyte, preDC and nonconventional DC which include monocyte-derived DC and plasmacytoid DC.

CD74 CD74−/− LDLR−/− ↓ 74% CD74, also known as invariant chain, acts as chaperone in endoplasmic reticulum and cell trafficking of the MHC II receptor. CD74 deficient mice have impaired antigen presentation and greatly reduced CD4+ and NK1.1+ cells in plaque as well as decreased IgG production in response to either MDA-LDL or bacterial HSP65 (1707). Would be expected to affect all populations of DC.

FLT3 FLT3−/− LDLR−/− ↑ 68% FLT3 (FMS-related tyrosine kinase 3 where FMS refers to feline McDonough sarcoma) is a cytokine receptor tyrosine kinase for cytokine FLT3L. FLT3 promotes proliferation of a CD103+ subset of conventional DC that were protective and promoted increased Treg (314).
The distinction between innate and adaptive immunity was formally first presented just over 20 years ago (844) and is now a guiding principle in immunology (1). Innate immunity refers to a system of inherited defenses activated by pattern recognition receptors (PRR) which bind pathogen-associated molecular patterns (PAMPs) that are invariant features of invading microbes. Innate responses need no prior exposure or “learning” or “memory” to be fully functional. Innate immune responses are found (at least to some degree) in virtually all multicellular organisms (836). Adaptive immunity is found only in jawed vertebrates and essentially involves activation of mature T and B lymphocytes. For an adaptive immune response to be mounted, a protein or peptide derived from a perceived invader must be recognized as foreign, and this signal must be paired with a pro-

### Table 7. Continued

<table>
<thead>
<tr>
<th>Gene</th>
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</tr>
</thead>
<tbody>
<tr>
<td>CCL17</td>
<td>CCL17E/E apoE−/−</td>
<td>↓50%</td>
<td>CCL17E/E &quot;E&quot; represents &quot;enhanced green fluorescent protein&quot; replacing then normal CCL17 allele by gene targeting. While CCL17 is a chemokine, it is included because of unique, cytokine-like effects on other cells. Apparently produced by a subset of conventional DCs that enter the plaque after onset of inflammation. Fewer macrophages and marked reduction in T-cells seen in lesions from CCL17-deficient mice. CCL17 was shown to restrain Treg proliferation, while CCL17E/E DC caused increased Treg proliferation and non-Treg CD4+ T-cell apoptosis. Similar results seen with CCL17E/E apoE−/− BMT into apoE−/− mice (1932).</td>
</tr>
<tr>
<td>CCR4</td>
<td>CCR4−/− BMT into LDLR−/−</td>
<td>NS</td>
<td>An additional receptor besides CCR4 was thought to mediate the effects CCL17 based on above results (1932). CCR4 unequivocally promotes CTL (CD8+ cytotoxic T lymphocytes), Th (CD4+), and Treg cell chemotaxis toward CCL17 producing DCs. CCR4 is involved in pulmonary inflammation from cigarette smoke (1491). CCR4 ligation can activate platelets and increases P-selectin expression. CCR4 antagonism was reported to inhibit Treg function in another study (533).</td>
</tr>
<tr>
<td>CCL22</td>
<td>in vitro anti-CCL22 antibody</td>
<td>NS</td>
<td>CCL22 is another ligand for CCR4. CCL22 is also known as MDC (monocyte-derived cytokine) and is produced by DC, macrophages, and NK/T and is induced by IFNγ. CCL22 antagonism had no effect on enhanced Treg proliferation (1932).</td>
</tr>
<tr>
<td>IFN-α</td>
<td>peritoneal injection of IFN-α into LDLR−/−</td>
<td>↑65%</td>
<td>IFN-α is a type I interferon produced by activated plasmacytoid DC that has strong inflammatory effects. Every other day injections of IFN-α increased plasma lipids modestly but significantly. Interpretation is therefore complicated (1014). A recent confirmation in apoE KO mice did not report a change in lipids (439).</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>daily injections of IFN-γ into LDLR−/−</td>
<td>↑78%</td>
<td>Similar properties as IFN-α. No increase in serum lipids in this study. IFN-γ increased macrophage adhesion to endothelium through increased CCL5 secretion by macrophages. Atherosclerosis was also increased in apoE KO mice with carotid collar placement. Increased atherosclerosis was seen despite increased IL-10 secretion as well (639).</td>
</tr>
<tr>
<td>IFNAR1</td>
<td>IFNAR1del BMT into LDLR−/−</td>
<td>↓34%</td>
<td>Cre-lox, myeloid-specific deletion of the main receptor for IFN-α/β (type I interferons). Involved in myeloid interferon signaling. Necrotic core size and apoptosis were particularly decreased. IFN-α induced macrophages showed increased CCR5 expression, CCL5 secretion, and endothelial adhesion despite higher IL-10 production. Endothelial adhesion was not inducible by IFN-γ in macrophages from STAT1−/− or IFNAR1del mice. IFN-β treatment increased atherosclerosis in apoE−/− and LDLR−/− mice. IFNAR1del mice had 70% reduction in plasma IL-6. (639).</td>
</tr>
<tr>
<td>BST2</td>
<td>Anti-BST2 antibody: in apoE−/− in LDLR−/− with carotid collar</td>
<td>↓50%</td>
<td>BST2 is a tetherin that can bind budding viruses. Its function remains obscure but anti-BST2 depleted pDC (plasmacytoid DC). (439). Why opposite results were seen with this using a similar pDC depletion approach remains unexplained (376).</td>
</tr>
</tbody>
</table>

Definitions are as in Table 1.

In **Table 7** is provided a list of cytokines manipulated by molecular methods as well as related genes affecting early stages of endothelial activation, leukocyte infiltration of the intima, other aspects of early inflammation, and their effects on atherosclerosis. Among the most important benefits of these studies has been clarifying the importance of innate versus adaptive immunity in atherosclerosis and the roles that specific types of leukocytes may play.

### B. Innate Immunity: An Overview

The distinction between innate and adaptive immunity was formally first presented just over 20 years ago (844) and is now a guiding principle in immunology (1). Innate immunity refers to a system of inherited defenses activated by pattern recognition receptors (PRR) which bind pathogen-associated molecular patterns (PAMPs) that are invariant features of invading microbes. Innate responses need no prior exposure or “learning” or “memory” to be fully functional. Innate immune responses are found (at least to some degree) in virtually all multicellular organisms (836). Adaptive immunity is found only in jawed vertebrates and essentially involves activation of mature T and B lymphocytes. For an adaptive immune response to be mounted, a protein or peptide derived from a perceived invader must be recognized as foreign, and this signal must be paired with a pro-
inflammatory innate immune response. Thus a “foreign” signal must be combined with an innate “danger” signal for adaptive immune responses to be triggered. On a molecular level, this elaborate control of adaptive immunity by innate responses is accomplished by an array of receptors which form an “immune synapse” between antigen presenting cells and antigen recognizing T cells. Antigen presenting cells include macrophages, dendrocytes, B-cells, and sometimes endothelial or other cells. In immune synapses, a series of costimulatory or coinhibitory receptors are found closely associated with the TCR. When an appropriately presented antigen binds to a TCR, a series of receptor-associated tyrosine kinases are activated, phosphotyrosine binding sites are created to which various adapters and signaling molecules attach, followed by activation of downstream signaling. Simultaneous activation of innate responses leads to upregulation of costimulatory receptors, and these further promote tyrosine kinase activity and downstream phosphorylations. In contrast, coinhibitory receptors activate highly effective phosphatases which quell the signal by removing the activating tyrosine and PI3 phosphate groups. Various cytokines may promote or inhibit adaptive immune responses.

Innate immune responses are triggered by transmembrane receptors, intracellular receptors, and secreted proteins. Transmembrane PRR can mount responses before cell infection. One class of transmembrane PRR, the Toll-like receptors, has already been presented. Other transmembrane PRR include scavenger receptors, C-type lectin receptors, and N-formyl Met-Leu-Phe receptors. (Bacteria make proteins with formyl-methionine as the initiating amino acid, rather than the methionine utilized by mammals and other higher organisms.) Intracellular PRR can be cytoplasmic or mitochondrial. Secreted PRR include collectins (e.g., mannoside-binding lectin), ficolins, and pentraxins (1, 1088). When secreted PRR coat invaders, they provide recognizable patterns to various innate receptors allowing phagocytosis and destruction. Toll-like receptors and scavenger receptors also recognize damage-associated molecular patterns (DAMPs), which occur endogenously without infection such as on apoptotic cells. These are particularly important in atherogenesis (1088).

Also to be included in the innate immune system are “natural antibodies” which are IgM antibodies coded by inherited, invariant DNA sequences and secreted spontaneously by a specific subset of B-cells (B1a cells) without prior exposure to any specific antigen or stimulation by T-cells. B1a cells are present and secreting their natural antibodies by birth or shortly thereafter, although increased secretion can be induced. Some natural antibodies bind “phosphocholine” which loosely refers to various phosphorylated choline motifs found in bacterial cell walls but also present in oxidized phospholipids (554, 728).

PRR that activate the transcription factors NF-κB, interferon regulatory factors (IRFs), and MAPK pathways can help trigger an adaptive immune response by stimulating both cytokine release and expression of transmembrane costimulatory receptors. Thus, when LPS binds TLR4 on dendritic cells (DC), macrophages, or endothelial cells, NF-κB is activated, TNF-α is secreted (peaking at ~1 h), followed by IL-1 release (peaking at 2 h) and chemokine secretion. Endothelial cells are stimulated to display selectins and adhesion molecules, while chemokines are trapped in their glycocalyx. Leukocytes are captured and move toward the site of inflammation, guided by a chemokine gradient. All this is in the realm of innate immunity. Macrophages summoned and activated by such an inflammatory stimulus, or activated DC, typically display costimulatory receptors and will activate T-cells if the antigens presented match the TCR of the T-cells present, thereby triggering an adaptive immune response.

Belonging to both innate and adaptive immunity are Fcγ receptors that can recognize antibodies bound to antigen. Some Fcγ receptors stimulate phagocytosis and cell activation while others mediate inhibition. The response depends on whether the receptor is linked to a coreceptor that contains an activating ITAM motif (such as FcγRIII) or contains an inhibiting ITIM motif in its cytoplasmic tail (e.g., FcγRIIb). As reviewed in TABLE 7, ITAM-containing FcγRI and FcγRIII were associated with increased atherosclerosis while FcγRIIb, with its ITIM linkage, was protective.

### C. Characteristic Cytokines and Responses by Cell Type

1. The mouse Ly6C<sup>hi</sup> monocyte subset preferentially gives rise to more inflammatory macrophages which become foam cells

Circulating monocyte subsets can be defined by cell-surface markers. High expression of the mouse cell-surface marker Ly6C<sub>hi</sub> (Ly6C<sup>hi</sup>) identifies the most prevalent monocyte subset. Ly6C<sup>hi</sup> monocytes also express CCR2, the receptor for MCP-1 (CCL2). Humans have an analogous subset (599). Ly6C<sup>hi</sup> monocytes are preferentially recruited to inflamed areas (including actively developing plaques), in part through MCP-1, and preferentially give rise to classically activated (M1) macrophages (see below) and foam cells (1721). In contrast, Ly6C<sup>lo</sup> monocytes lack CCR2 but display more CX3CR1 (the fractalkine receptor) and seem adapted to crawl along endothelial surfaces for general surveillance, with preferential recruitment to become tissue resident macrophages, myeloid DC (dentritic cells), and alternatively activated (M2) macrophages (see below). In the circulation, Ly6C<sup>hi</sup> monocytes can also be converted to Ly6C<sup>lo</sup> monocytes. Interestingly, during the course of atherogenesis in both ApoE<sup>−/−</sup> and LDLR<sup>−/−</sup> mice, progenitors of Ly6C<sup>hi</sup> monocytes progressively relocate from bone...
marrow to splenic red pulp. These cells give rise to M1 type macrophages and eventually ingest lipid to become foam cells (1493).

2. Macrophage phenotypes: artificial but useful constructs based on in vitro cytokine, PAMP, and DAMP exposure

Incubation of macrophages with different PAMPs, DAMPs, cytokines, scavenger receptor ligands, and various lipids is followed by characteristic changes in expression of surface markers, cytokine and signaling molecules, as well as changes in lipid handling and propensity toward foam cell formation. The resulting macrophage phenotypes may have apparently opposite functions (such as pro-inflammatory versus anti-inflammatory, or tissue repair promoting). While recognition of macrophages with widely differing functions or phenotypes is useful, macrophages in vivo actually display plasticity in modulating between not only these polarized phenotypes but activation states with intermediate features, depending on stimuli encountered (1237).

Classical macrophage activation involves incubation with IFN-γ, which primes macrophages so that subsequent TLR activation upon exposure to various organisms such as *Lesbmania* or *Toxoplasma* parasites or the intracellular bacteria *Listeria monocytogenes* or *Mycobacterium tuberculosis* triggers vigorous activation and killing. Without prior IFN-γ exposure, these organisms can survive intracellularly in macrophages (1261). IFN-γ plus LPS (to activated TLR4) or IFN-γ plus TNF-α triggers similar activation, suggesting JAK-STAT signaling from IFN-γ together with activation of NF-κB mediate classical macrophage activation (1237). Classically activated macrophages are said to display the M1 phenotype, so named because IFN-γ is characteristically secreted by activated Th1 cells (but also by stimulated natural killer or CD8+ cytotoxic lymphocytes). In addition, M1 macrophages secrete cytokines like IL-12 that further promote the Th1 response. M1 macrophages secrete TNF-α, type I interferons such as IFN-β (with further autocrine activation of these macrophages by both TNF-α and IFN-β), IL-1β, IL-6, IL-12, IL-23, CCL15, CCL20, CXCL9, CXCL10, and CXCL11. M1 macrophages also express increased MHCII, costimulatory receptors, NOX2 and ROS, NO from iNOS, arginase-2 and prostanoids from increased COX2 expression (153, 1237).

Further efforts have been made to characterize the separate and combined effects of IFN-γ priming and LPS stimulation (213). In M1 activation, both p38 and ERK activation appear to be important to stimulate MNK1 which then phosphorylates the transcription factor eIF4E, allowing full transcription of hundreds of genes. Signaling from IFN-γ primes M2-type activation (791, 1999). Interestingly, other than through STAT1 is stimulated after IFN-γ stimulation of the MEKK4/MEK6/p38 cascade (698). The adapter protein Cbl may bind to the activated IFNGR followed by tyrosine phosphorylation by JAK with subsequent activation of PI3K and downstream stimulation of the PKB/Akt cascade. PKB provides anti-apoptotic effects and can support subsequent protein synthesis through mTOR activation. In addition, PIP3 formation promotes activation of PKCδ through PDK1 as well as NOX2 activity. JAK1 and JAK2 also activate PLCγ with release of IP3, DAG, and IP3-mediated calcium release. Calcium binds calmodulin followed by CaMKII activation. Both PKCδ and CaMKII can serine phosphorylate S727 on STAT1 which is required for full transcriptional activity of the STAT1 dimer (418, 1420).

Further efforts have been made to characterize the separate and combined effects of IFN-γ priming and LPS stimulation (213). In M1 activation, both p38 and ERK activation appear to be important to stimulate MNK1 which then phosphorylates the transcription factor eIF4E, allowing full induction of interferon regulatory factor 8 (IRF8). IRF8, in turn, is a transcription factor for such M1 genes as IL12a, IL12b, and iNOS but may also be involved in M2 activation. IFN-γ may also increase expression of IRF8.

There is cross-talk between TLR4 and NOTCH signaling. NOTCH signaling is an ancient, conserved signaling pathway that controls cell proliferation and differentiation of many cell types, particularly during embryogenesis. NOTCH is actually not an abbreviation but refers to a characteristic notch in the wings of fruit flies with a NOTCH receptor mutation. The pathway noted here illustrates NOTCH signaling generally, but its activation as part of M1 activation was not suspected until recently. Apparently, activated macrophages express a cell-surface bound NOTCH ligand which when bound to the NOTCH1 receptor on a neighboring macrophage leads to ADAM10-mediated cleavage of the extracellular domain of the NOTCH1 receptor. Immediately thereafter, there is cleavage of the NOTCH intracellular domain just below the inner leaflet of the cell membrane by the action of γ-secretase. This two-step proteolysis releases NICD1 (NOTCH intracellular domain 1) which translocates to the nucleus and binds its target RBP-J, changing RBP-J from a repressor to an active transcription factor. Among the effects is augmented MAPK activation through increased IRAK2 expression, as well as increased induction of IL-6. RBP-J also actively suppresses M2-type activation (791, 1999). Interestingly, other...
targets of RBP-J are the transcription repressors Hes1 and Hey1 which act as feedback inhibitors of IL-6 and IL-12 transcription. IFN-γ inhibits Hes1 and Hey1 transcription, allowing unaltered production of IL-6 and IL-12.

Incubation of macrophages with the Th2 cytokines IL-4 and/or IL-13 or GM-CSF with IL-4 was first proposed to cause “alternative activation,” leading to the M2 phenotype (641, 642). IL-4 and IL-13 signaling are characterized by downstream LXRα and PPARγ activation (1324). M2 macrophages are important for fighting parasitic infections but are also referred to as “wound healing macrophages” and appear to be involved in inflammation resolution. Some consider the parasite-killing but wound-resolving M2 macrophages as a separate phenotype from IL-10 producing “regulatory macrophages” or Mreg which develop after exposure to IL-10 (1237). Alternatively activated M2 macrophages express increased MHCII, the macrophage mannose receptor, dectin-1, arginase-1, and secrete IL-1Ra (IL-1 receptor antagonist), CCL13, CCL17, CCL18, CCL22, and CCL23 (which are chemoattractive for Th2 lymphocytes and other cells), IGF1 (which stimulates fibroblast proliferation and survival), and display increased endocytosis activity and parasite killing but greatly decreased iNOS. They also secrete several proteins that bind the extracellular matrix and promote would healing and can secrete the highly anti-inflammatory IL-10 and TGFβ (642, 1237).

IL-4 and IL-13 can both signal through the IL4RA/IL13RA2 receptor, a JAK-STAT receptor that activates STAT6 (153). In addition, IL-13 has nonredundant effects by uniquely signaling through IL13RA2. Rather than a decay receptor as previously thought, IL13RA2 appears to have greater anti-inflammatory effects than IL4RA (258). Transplant of IL-13-deficient bone marrow into LDLR-null mice clearly increased atherosclerosis (258), whereas effects of IL-4 deficiency were more ambiguous (see Table 7). GM-CSF, IL-4, and IL-13 all induce increased expression of PPARγ which seems to characterize the M2 phenotype (194, 1324, 1601). In addition, transcription factors KLF4, LXRα, PU.1, and IRF8 are also key in M2 differentiation. For example, LXRα was found to bind the promoter of IRF8 to increase its transcription while IRF8 and PU.1 interact at a composite response element in the promoter of arginase-1 (1262, 1435).

Interestingly, M2 macrophages are first to appear in developing plaques in ApoE-deficient mice. M1-type macrophages only appear in more advanced, progressing lesions associated with greater inflammation (900). Human carotid atherosclerotic lesions were also noted to display a mixture of M1 and M2 phenotypes (194). The early appearance of M2 macrophages is consistent with the view that the usual, steady-state status of tissue macrophages is anti-inflammatory and immunosuppressant, accompanied by copious secretion of IL-10 (what some might call Mreg macrophages). Such a state allows normal housekeeping activities such as efferocytosis to proceed without inflammation (1262). Immunosuppressive Mreg type macrophages are particularly important in the gut where exposure to foreign antigens from digested food or bacteria is common. M2 activation may be viewed as an early, less aggressive activation with perhaps more rapid transition to resolution. M1 activation appears to be reserved for the most vigorous assaults, triggered by cooperation between IFN-γ-producing Th1 and activation of TLRs or other pathogen pattern recognition receptors. A number of signals promote reversion of M1 macrophages to the less inflammatory phenotypes such as various lipid ligands (such as electrophilic nitro fatty acids which activate PPARγ), certain signals from apoptotic cells, and interestingly, ApoE binding its own receptor expressed on macrophages (APOER2, also known as LRP8), or VLDLR (the VLDL receptor) (99, 1448).

Several other macrophage phenotypes have been described. Incubation of macrophages with oxidized phospholipid stimulates Nrf2 activation with increased expression of redox control genes (such as HMOX1) with an overall different gene expression pattern than either M1 or M2 macrophages. These so-called Mox macrophages had decreased phagocytic and chemotactic capacity. In aortic atheroma from LDLR-null mice, 39% of the macrophages appeared to have an M1 phenotype, 21% were M2, and 30% had an Mox phenotype (872). The Mres resolving phenotype may be thought of as a subtype of M2, developing after exposure to resolvins with increased cAMP (12). The proposed M4 phenotype has a number of features in common with M1 and develop in response to CXCL4 (621). M4 macrophages suppress expression of protective CD163 and HMOX1 (622). KO of CXCL4 decreased atherosclerosis in ApoE-deficient mice (1521).

Among the most interesting of macrophage phenotypes is Mhem (199). Mhem develop upon exposure of nonpolarized, peripheral monocytes to hemoglobin/haptoglobin complexes (HbHp) which bind CD163, the macrophage HbHp scavenger receptor. Red cell phagocytosis also contributes to the Mhem phenotype. Resulting signaling leads to a marked upregulation of CD163, HMOX1, macrophage mannose receptor, ABCA1, ABCG1, and IL-10, together with reduced expression of MHCII, SR-A, and CD36. IL-10 was necessary for autocrine stimulation of the positive feedback loop leading toward differentiation of this phenotype (199, 200). Moreover, such cells did not accumulate lipid yet were clearly present in human plaques that had undergone intraplaque hemorrhage. The Mhem were found next to but were distinct from foam cells (200, 201).

Intracellular signaling leading to the Mhem phenotype has been partially worked out. Intracellular heme accumulates
after HbHp-CD163 uptake or erythrocyte phagocytosis with lysosomal degradation. Mild to moderate oxidative stress ensues, apparently activating Nrf2 and possibly the transcription factor ATF1. PKA activation may also be involved. Active PKA can activate ATF1 as well. Additionally, heme or even the iron-free protoporphyrin ring of heme can inactivate a transcription repressor, Bach1 (Btb And Cnc Homology 1), which blocks ARE sites activated by Nrf2 (1144). Both Nrf2 and ATF1 then induce HMOX1, ferritin, and ferroportin. Active ATF1 additionally increases expression of LXRxβ, followed by LXRxα and PPARγ activation, with downstream induction of ABCA1 and ABCG1 and presumably other markers of Mhem such as IL-10 (199, 201).

Accumulating iron further promotes translation of ferritin and ferroportin and blocks translation of transferrin receptors through IRP/IRE control. Iron response elements (IREs) are stem/loop structures in mRNA which occur in 5' or 3' untranslated regions of many iron-related genes. In low iron states iron regulatory protein 1 or 2 (IRP1/2) binds IREs. In the proximal 5' region, IRP binding to an IRE blocks ribosome attachment and polysome formation, thereby inhibiting protein translation. In the distal 3' region, IRP binding to IREs leads to stabilization of the mRNA, apparently by blocking RNase recognition sites, resulting in increased translation. This elegant control allows 1 protein (an IRP) to control expression of different target proteins in opposite directions. When cellular iron is high, the IRP proteins bind iron which promotes their ubiquitination and degradation. For the proteins of concern here, as cellular iron increases IRP1/2 is bound by iron and degraded leaving 5' IREs in ferritin and ferroportin unbound with increased translation, thereby providing safer intracellular storage (by ferritin) and more transport of iron out of cells (by ferroportin). At the same time, unoccupied IREs in the 3' region of transferrin receptor mRNA lead to increased mRNA lysis and downregulation of transferrin receptors that would otherwise further increase uptake of iron into the cell.

Mhem is a macrophage specialized in taking up HbHp complexes and effete red blood cells while rapidly excreting accumulated free iron through ferroportin without excess production of ROS. At the same time, it suppresses surrounding inflammation and promotes repair by secreting IL-10. Such polarization would be particularly advantageous in areas of hemorrhage or traumatic wounds. Furthermore, this Mhem phenotype actively mobilizes intracellular cholesterol to HDL through upregulated ABCA1 and ABCG1, thereby elegantly dealing with the cholesterol derived from phagocytosed erythrocytes while being resistant to additional lipid accumulation by downregulation of SR-A and CD36. Thus handling of heme and iron by Mhem is physically segregated from the lipid accumulation and superoxide production that occurs in M1-type macrophages. Such segregation avoids an otherwise highly inflammatory setting since both heme and hemoglobin are strong oxidizers of LDL and cellular lipids as are the ROS generated by M1 macrophages, especially in the presence of iron. Excess lipid oxidation would generate ligands for TLR, LOX1, and RAGE as well as cause direct damage to cellular proteins and DNA, further exacerbating the inflammation.

Safe storage of iron in ferritin is important in Mhem since free iron catalyzes the following reactions resulting in production of highly reactive hydroxyl radical:

\[
\text{Fe}^{3+} + \text{O}_2^- \rightarrow \text{Fe}^{2+} + \text{O}_2
\]

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \cdot\text{OH} + \cdot\text{OH} \quad \text{(Fenton reaction)}
\]

with the following net effect in the presence of iron:

\[
\text{O}_2^- + \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + \cdot\text{OH} + \cdot\text{OH} \quad \text{(iron catalyzed Haber-Weiss reaction)}
\]

A highly pro-inflammatory effect might therefore be expected if increased iron accumulated after the stimulation of M1-type macrophage activation (with increased superoxide production already present).

An interesting example of inhospitable iron accumulation in previously activated macrophages occurs with heme iron overload in chronic venous stasis ulcers, resulting in an unrestrained M1-like phenotype together with increased production of the highly reactive hydroxyl anion and peroxynitrite. The presence of such highly activated macrophages were found to impair wound healing. Some M2 or Mhem markers were expressed as well, suggesting an incomplete switch to the Mhem phenotype (1634).

In another study, macrophages initially activated by oxLDL showed even greater M1-type activation after erythropagocytosis. This activation was inhibited by iron chelators and markedly exaggerated by hepcidin, the small, hormone-like peptide that binds the extracellular side of ferroportin, promoting its internalization and degradation. Thus the effect of hepcidin in macrophages is to reduce iron release and increase the intracellular iron load. Additionally, hepcidin production by macrophages was transiently upregulated by inflammation (for example, after exposure to oxLDL). This might be expected since hepcidin production by hepatocytes is well known to be upregulated by IL-6 through production of active STAT3 (585). The result was highly activated, inflammatory macrophages that were both lipid and iron loaded, possibly reflecting a macrophage phenotype that would be expected in advanced atherosclerotic lesions with intra-plaque hemorrhage. In the same study, carotid arteries were treated with viral vectors to transgenically overexpress hepcidin in ApoE-deficient mice.
Plaques that developed were only slightly larger but clearly more vulnerable and showed substantially greater accumulation of oxLDL and greater expression of inflammatory cytokines including IL-6, MCP-1, TNF-α as well as MMP2. In contrast, hepcidin knock-down greatly decreased oxLDL and the cytokines in plaque macrophages (1025). In the setting of an advanced atherosclerotic plaque with secondary intraplaque hemorrhage, the potentially anti-inflammatory effects of heme are probably “too little, too late.” Instead, the increased cellular iron may just contribute to the inflammation (199).

Reduced inflammatory responses by iron-depleted macrophages have also been reported. In mice with hepcidin deficiency due to KO of the hemochromatosis gene (HFE), an oral *Salmonella* challenge resulted in much less intestinal inflammation (1901). Macrophages from these HFE−/− mice showed increased ferroportin expression and decreased intracellular iron with reduced inflammatory responses when challenged in vitro with *Salmonella* or LPS. These diminished inflammatory responses to *Salmonella* or LPS were reproduced in wild-type macrophages by iron depletion with iron chelators or by overexpression of ferroportin.

These studies provide insights into why hemochromatosis has not been associated with an increased risk of coronary atherosclerosis, and may even decrease risk in some circumstances, despite whole-body iron overload (1702). Elucidation of the hepcidin-ferroportin axis obtained over the last 15 years is key to this understanding. Normally, hepatocytes sense total body iron status by their own iron content and by the amount of plasma holotransferrin (transferrin carrying its full complement of 2 iron atoms) encountered by transferrin receptor 2 (TFR2) on hepatocytes. Rising intracellular hepatocyte iron causes, by an unknown mechanism, an increase production and extracellular release of BMP6. In autocrine fashion, BMP6 binds a BMPR (BMP receptor) which serine phosphorylates SMAD1/5/8. Active SMAD1/5/8 then combines with SMAD4 and translocates to the nucleus to increase hepcidin synthesis. Hepcidin is released and circulates around the body, binding to and promoting degradation of ferroportin. The activity of the BMPR is greatly modified by the coreceptor hemojuvelin (HJV) which, in turn, forms a complex with HFE (the hemochromatosis protein) and TFR2 when TFR2 is occupied by holotransferrin (371, 491, 585). Normally, iron binding sites on circulating transferrin are only ~30–35% occupied. HFE, in some way, acts to further enhance BMP6 signaling when TFR2 is bound. Other factors that modify hepcidin transcription are IL-6 acting by STAT3 activation which increases hepcidin transcription, while decreased hepcidin expression is independently mediated by bone marrow-derived signaling proteins that are released in the face of anemia, and by hypoxia through HIF signaling proteins.

Hereditary hemochromatosis is caused by inherited defects in hepcidin production. By far the most common cause of hemochromatosis is homozygosity for a common HFE mutation (C282Y) present in heterozygous form in nearly 10% of northern European populations. The absence of HFE leads to greatly reduced hepcidin production regardless of liver iron status. Hepcidin normally travels to the gut and causes internalization and degradation of ferroportin on intestinal brush-border cells, thereby inhibiting release into the bloodstream of the iron absorbed by these cells. Thus, when iron stores are sufficient, iron that is internalized by enterocytes from the gut lumen is simply lost back into the intestine as these cells are sloughed off. Hepcidin also reduces transferrin saturation by blocking iron release through ferroportin by reticuloendothelial macrophages, which actively remove and ingest aged red blood cells. In hemochromatosis, abnormally reduced hepcidin allows unrestrained iron absorption through ferroportin in enterocytes. This leads to iron overload in liver, pancreas, and myocardium. However, hepcidin deficiency and continued high ferroportin expression in macrophages leads to unregulated release of iron from these cells. Macrophages thus become essentially iron deficient in hemochromatosis despite the iron overload in the liver and certain other tissues. Adipocytes also express ferroportin and show marked reduction in intracellular iron in hemochromatosis, interestingly with improved insulin sensitivity and increased production of adiponectin (573). As noted above, relatively iron-depleted macrophages from HFE−/− mice appear to be less responsive to inflammatory signals. In addition, an iron-deficient diet, which naturally leads to marked reductions in hepcidin, reduced atherosclerosis in ApoE KO mice and was associated with reduced LDL oxidation in the lesions (995).

Recently, the effect of hepcidin reduction by pharmacological means was tested for its affect on atherosclerosis in ApoE-deficient mice (1526). A small molecule inhibitor of SMAD1/5/8 activation (LDN 193189) was used to reduce hepatic expression of hepcidin. Macrophage ferroportin expression was increased and atherosclerosis area in the aortic sinus and aorta were decreased 43–50%. Furthermore, lesion macrophages had less lipid, expressed more ABCA1 and much more ABCG1. In vitro, peritoneal macrophages from the treated mice demonstrated greater cholesterol efflux together with reduced hydrogen peroxide production. These in vitro macrophage findings were reversed by incubation with hepcidin. Certainly, before such a compound could be considered for human use, longer term testing for effects on iron homeostasis in vulnerable tissues such as liver, pancreas, and myocardium would be necessary.

### 3. Other cytokines associated with macrophages

As suggested by the above discussion, certain cytokines are classically associated with specific leukocytes. Those studied for effects on atherosclerosis are included in Table 7.
Besides atherogenic TNF-α and IL-1, macrophages secrete IL-6, IL-12, and IL-18 in response to LPS (3 h peak). IL-6 causes synthesis of acute phase reactants (and hepcidin) in the liver but was only variably associated with atherosclerosis in genetic models (see Table 7). IL-12 and IL-18 were atherogenic. They stimulate T-helper 1 cells (Th1) to secrete IFN-γ. KO of IFN-γ or its receptor IFNGR1 decreased atherosclerosis (see Table 7 under Th1 cells). IL-12 also stimulates cytotoxic CD8+ T lymphocytes (CTL) and NK cells to increase their cytotoxic activity. Other interleukins secreted by macrophages include IL-23 and IL-27, but their effects on atherogenesis have apparently not been studied. Growth differentiation factor 15 (GDF-15) is an atherogenic cytokine in the TGF-β superfamily. GDF-15 is secreted by macrophages and promotes macrophage accumulation in a CCR2-dependent manner (392).

M-CSF is a chemokine-like cytokine whose cognate receptor is CSF1R (see Table 6). M-CSF production is induced by oxLDL in endothelial cells and VSMC. It appears to be key for growth and survival of macrophages. M-CSF also stimulates production of other cytokines and may induce transformation of VSMC into foam cells (918). Deficiency of M-CSF results in macrophage-deficient mice which also lack osteoclasts and are osteopetrotic (op). The oplot mouse is homozygous for a naturally occurring base pair insertion in M-CSF leading to a premature stop codon with marked reduction in M-CSF. Oplot mice are highly resistant to atherosclerosis. M-CSF<sup>−/−</sup> also showed substantial reduction of atherosclerosis as did KO of its receptor (see Table 6). Almost all the effect of M-CSF on atherosclerosis was attributable to vascular wall production or other cells other than bone marrow derived (1602).

Several miscellaneous cytokine-like macrophage activators are also reviewed in Table 7. Those increasing atherosclerosis include osteopontin (OPN), macrophage migration inhibitory factor (MIF), the NOTCH ligand DLL4, and netrin 1 (Ntn1), which increases atherosclerosis by possibly causing macrophage retention in plaques. The tetrahydrocannabinol receptor CB2 has neutral effects. Protective GRK5 is not a cytokine, but it phosphorylates proinflammatory receptors CCR2 and CSF1R, marking them ubiquitination and degradation.

Certain lipid mediators of inflammation may be considered complementary to chemokines or cytokines. For example, S1P acting through S1PR2 increased atherosclerosis while S1PR3 had no effect. Eicosanoids can have profound effects on macrophages and other leukocytes with both cytokine and chemokine-like effects. PGE<sub>2</sub>, produced by macrophages themselves, can bind Gα<sub>q</sub>-coupled receptors on themselves or nearby macrophages and promote survival, leading to larger plaques. The EP2 receptor had little effect, but KO of EP4 reduced atherosclerosis lesion size (see Table 7). Another group of lipid mediators of inflammation, the leukotrienes, have received considerable attention as discussed in the following section.

Much that is said regarding macrophages might also apply to dendritic cells. Indeed, one dendritic subtype is derived from monocytes. However, because dendritic cells appear to be the first foam cells, they are discussed in section IXA. Genes that have been manipulated in regard to dendritic cells remain, however, at the end of Table 7.

4. 5-LO and the leukotrienes

As depicted in Figure 15, the leukotrienes are products of lipoxygenase enzymes that can act as lipid counterparts to the peptide cytokines and chemokines. As such, they are essentially amplifiers of early inflammation. The leukotrienes are strongly pro-inflammatory eicosanoids synthesized from arachidonic acid (AA) primarily by macrophages and other activated leukocytes. Various inhibitors of the pathway have long been marketed for treatment of asthma. The initial step in leukotriene synthesis occurs when free AA, released by cPLA<sub>2</sub>, is presented by the adapter 5-lipoxygenase activating protein (FLAP) to the enzyme 5-lipoxygenase (5-LO). This step occurs on the nuclear envelope with localization of 5-LO provided by FLAP. AA is converted to 5-LO to the unstable product leukotriene A<sub>4</sub> (LTA<sub>4</sub>) which is converted to LTB<sub>4</sub> by LTA<sub>4</sub> hydrolase (LTA<sub>4</sub>H). LTB<sub>4</sub> is then transported out of the cell by a specific transporter. Alternatively, LTA<sub>4</sub> can be converted to LTC<sub>4</sub> by conjugation with glutathione through the action of LTC<sub>4</sub>S (LTC<sub>4</sub> synthase). LTC<sub>4</sub> is then transported out of the cell and further acted on by extracellular peptidases to produce LTD<sub>4</sub> and LTE<sub>4</sub>. LTC<sub>4</sub>, LTD<sub>4</sub>, and LTE<sub>4</sub> are referred to as cysteinyl-LTs and bind to CysLT<sub>1</sub>, CysLT<sub>2</sub>, or CysLT<sub>E</sub> which are Gα<sub>q</sub>-coupled GPCRs. These receptors promote endothelial permeability, among other pro-inflammatory effects. Anti-asthma drugs montelukast, zafirlukast, and pranlukast all inhibit the CysLT<sub>1</sub> receptor. However, LTB<sub>4</sub> is actually the most inflammatory leukotriene through binding its receptors BLT1, a Gα<sub>q</sub>-coupled GPCR found mainly on hematopoietic cells but also on activated endothelium such as that overlying atherosclerotic lesions. A second LTB<sub>4</sub> receptor, BLT2, is widely expressed.

As shown in Figure 15, LTB<sub>4</sub> acting through BLT1 amplifies early inflammatory signaling. LTB<sub>4</sub> is clearly chemotactic for neutrophils and macrophages, in part by reducing cAMP levels in migrating cells, similar to chemokines. Of note, elevation of cAMP by various agents inhibits cell migration generally, apparently by PKA phosphorylation of MLCK with a reduction in myosin light-chain phosphorylation and other actions to inhibit lamellipodia formation and extension (292, 1076). Other inflammation-amplifying effects of LTB<sub>4</sub> were recently demonstrated in the setting of intermittent hypoxia (1031). Exposure of ApoE KO mice to intermittent hypoxia clearly increases atherosclerosis, possibly by increasing ROS generation by mitochondria from...
spillover and promiscuous loss of free electrons from the electron transport chain when acceptor O₂ is limited, followed by greater activation of NF-κB, p38, and JNK. Interestingly, LTB₄ and BLT1 were also increased by intermittent hypoxia, while KO of BLT1 eliminated the excess atherosclerosis associated with intermittent hypoxia. Further studies showed that BLT1 ligation by LTB₄ upregulated MAPK and NF-κB activity, resulting in increased transcription of S-LO, LTA₄H, and MCP-1, a positive feedback loop. At the same time, foam cell formation was promoted by increasing CD36 and SRA expression while macrophage differentiation was also stimulated. BLT1 signaling also promotes secretion of cytokines, MCP-1, and MMPs in other leukocytes as shown in Figure 15, further amplifying the inflammatory cascade.

Additional proinflammatory signaling is mediated by BLT1. In macrophages, and presumably in activated endothelium as well (as shown in Figure 15), activation of BLT1 is thought to lead to β-arrestin binding to the cytoplasmic side of the BLT1 receptor followed by Src activation. Src generally provides phosphotyrosines on scaffolds or adapter proteins for subsequent binding and activation of downstream effectors. In the case of BLT1 signaling, this action appears to result in JAK2-STAT1 signaling. Active STAT1 dimers were found to act as transcription factors to stimulate MyD88 transcription which would facilitate NF-κB activation through most TLR as well as IL-1R. Go₁ signaling through BLT1 also blocked SOCS1, a major inhibitor of JAK2 (1589).

Atherosclerosis was clearly reduced by blocking BLT1 either by genetic deletion (as in the intermittent hypoxia model noted above as well as in other studies of genetically hyperlipidemic mice), or by pharmacological inhibition of BLT1. Surprisingly, however, most of these studies showed effects only at early time points with little or no atherosclerosis reduction at later time points (1428) (see Table 7). Inhibition of CysLT₁ with montelukast also decreased atherosclerosis in hyperlipidemic mice in a relatively short-term (16-wk) study (1428). Follow-up of a very large Swedish drug registry found only suggestive effects for prevention of stroke or MI associated with montelukast use (824).

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In comparison to 5-LO, the physiological function of 12/15-LO is less clear, yet it is one of the most studied genes in atherosclerosis research. While there are several enzymes with lipoxygenase activity that can add peroxyl groups to the 8, 12, or 15 positions of AA, the enzyme of interest here is variously called leukocyte-type 12-LO (in mouse), 15-lipoxygenase-1 (in human), or simply 12/15-LO (567). 12/15-LO introduces a peroxyl group at the 12 or 15 position of AA, yielding 12-S-HPETE or 15-S-HPETE (hydroperoxyeicosatetraenoic acid). This transient product is acted on by cellular peroxidases to yield the more stable 12- or 15-HETE. In the mouse, the 12-LO activity predominates, whereas in rabbits and humans, 15-HETE is the main product. When acting on linoleate (which can include cholesterolinolate in LDL supplied in an incubation medium with intact macrophages), the main product is 13-HPODE with lipoxygenase activity that can add peroxyl groups to the 8, 12, or 15 positions of AA, the enzyme of interest here is variously called leukocyte-type 12-LO (in mouse), 15-lipoxygenase-1 (in human), or simply 12/15-LO (567). 12/15-LO introduces a peroxyl group at the 12 or 15 position of AA, yielding 12-S-HPETE or 15-S-HPETE (hydroperoxyeicosatetraenoic acid). Secretion of IL-12 by activated macrophages appears to be dependent on 12/15-LO as well as late resolution.

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12/15-LO, some with correlation between atherosclerosis and urinary levels of isoprostanes (2089) or inhibition of atherosclerosis with high doses of vitamin E only in mice having intact 12/15-LO (though the vitamin E may have inhibited the enzyme itself) (2092). However, in a subsequent study, whole body KO versus BMT of 12/15-KO cells into wild-type mice equally reduced atherosclerosis (807). Furthermore, this study showed that urinary isoprostane levels were the same in 12/15-LO replete mice transplanted with either 12/15-LO-deficient or wild-type bone marrow, but atherosclerosis reduction was seen only with 12/15-LO deficiency in hematopoetic cells. These results suggest a more specific signaling role, particularly in macrophages. These investigators went on to show in a two-chamber experiment that the medium from wild-type macrophages incubated with native LDL (not oxidized or otherwise modified) in the top chamber markedly increased subsequent test monocyte attachment to endothelial cells cocultured in the bottom chamber. Thus, even though only fluid from the incubated macrophages could bathe the endothelial cells, it still transmitted one or more endothelial activating substances. In this same experiment, macrophages from 12/15-LO KO mice did not induce endothelial monocyte attachment (807). These results suggested a specific product of 12/15-LO was acting as a soluble cytokine-like molecule.

The notion that 12/15-LO seeds lipoproteins with oxidized fatty acyl free radicals which propagate further free radical damage was largely dispelled by a recent study. Instead, fatty acids in intact cholesteryl esters derived from intracellular pools or external LDL can be directly oxidized by 12/15-LO, followed by hydrolysis of the cholesteryl ester and reesterification of the oxidized fatty acids into phospholipids. The stereospecificity of the products (12-S-HETE and 12-S-HODE) showed that the oxidized fatty acids were generated enzymatically with minimal nonspecific, spontaneous oxidation (808).

If 12/15-LO generates specific signaling molecules, what are they and what is the nature of their effects? 12-HETE generated by 12/15-LO was shown to critically affect monocyte adhesion to aortic endothelium by increasing active RhoA, PKCα, and NF-κB leading to increased cell-surface expression of ICAM-1 (173). However, where the 12-HETE is generated, whether in macrophages, other leukocytes, or activated endothelial cells could not be shown. Presumably, the BMT studies reviewed above suggest hematopoetic cells as the normal primary source. Perhaps this explains the severe atherosclerosis seen in transgenic mice with endothelial overexpression of 12/15-LO (170, 173, 715, 1469). These investigators showed that 12/15-LO stimulated the presentation of fibronectin by endothelial α5β1 integrins on the apical (luminal) side of the cells, leading to monocyte-specific capture through monocyte α5β1 integrins which recognize fibronectin (1469). Whether 12/15-LO is normally expressed in activated endothelial cells remains a question, however. Incubation of endothelial cells with high glucose, as might be seen in diabetes, led to an increase in 12-S-HETE production and a similar presentation of fibronectin by endothelial integrins which appeared to be dependent on 12/15-LO expression (1395, 1396). Interestingly, both 12/15-LO products and ANG II can stimulate VSMC to produce fibronectin (1034).

Recently, the orphan GPCR GPR31 was found to be a specific 12-S-HETE receptor. In endothelial cells, GPR31 ligation with 12-S-HETE increased MAPK and NF-κB activity (676). GPR31 signaling may explain the previously hypothesized GPCR which appeared to mediate endothelial effects of 12-HETE, including activation of PKCa, RhoA, NF-κB, and increased ICAM-1 expression (as shown in **Figure 15**) (173, 567). Whether this receptor can explain both the paracrine and apparent autocrine effects of 12-S-HETE remains to be explored. Another group identified specific Src activation followed by cell migration in VSMC stimulated with 12-S-HETE (1468). Such signaling might be expected if GPR31 ligation led to PYK2 activation. 12/15-LO was found to promote proliferation of VSMC and neointimal formation after arterial injury (407). 12-HETE was also recently reported to promote ER stress in adipocytes (338).

The above paragraphs briefly summarize the apparent pro-inflammatory, proatherogenic effects of 12/15-LO. Thus, in activated cells, this enzyme appears to produce 12-S-HETE which may act through a specific GPCR to activate MAPK and NF-κB with markedly increased endothelial expression of adhesion molecules. KO of 12/15-LO reduced atherosclerosis in most studies while nonphysiological transgenic overexpression, especially in endothelial cells, markedly increased atherosclerosis. If these were the only effects, the discussion of this intriguing enzyme could have been much abbreviated. However, newer studies reveal a major role for 12/15-LO in the resolution of inflammation. Thus 12/15-LO can hydroxylate LTA₄ produced by S-LO, on carbon 15 (or S-LO can act on 15-HETE) to yield the anti-inflammatory lipoxin LXA₄. Resolvins, protectins, and maresins are similar to lipoxins but are derived from n-3 fatty acids. Each of these pro-resolving lipids acts through its own GPCR (1592). The importance of 12/15-LO in inflammation resolution is clearly demonstrated in mouse arthritis models, where 12/15-LO KO results in severe, uncontrolled, prolonged inflammation with increased tissue damage (954). Interestingly, PGE₂ production, primarily by COX-2, was essential for the switch to resolution induced by LXA₄ in a mouse arthritis model (272). Anti-inflammatory effects of lipoxins include stimulation of macrophage differentiation into inflammation-resolving subtypes (such as M2) having greater ability to clear apoptotic debris (951), reduced cytokine production, decreased endothelial activation, and other ef-
fecteds (1592). Furthermore, phagocytosis of apoptotic cells may provide the appropriate precursor(s) (for example, LTA₄ from apoptotic neutrophils) as well as other triggers for the switch to greater LXA₄ synthesis in macrophages. Thus the accumulation of lipoxin precursors during inflammation may help stimulate the later increased production of lipoxins.

Considering these inflammation resolving effects, it may be less surprising that several studies have reported evidence for atherosclerosis protection by 12/15-LO. Transgenic WHHL (Watanabe heritable hyperlipidemic) rabbits (an LDLR⁻/⁻ model) which overexpressed human 15-LO were protected from atherosclerosis (1613). Later, macrophage-specific transgenic overexpression of the human 15-LO in ApoE null mice led to marked increase in production of LXA₄ and a reduction in atherosclerosis when the mice were fed a low-fat chow diet (1197). Furthermore, BMT of 12/15-LO deficient cells into ApoE-deficient animals fed a low-fat diet increased atherosclerosis compared with BMT of 12/15-LO⁺/⁺ cells, again suggesting protection mediated by 12/15-LO (1197). In these studies, the low-fat chow diet appeared to be of key importance. Thus the same group using the identical transgenic and BMT mouse models reported later that the apparently beneficial effects of 12/15-LO were essentially reversed by feeding a high-fat, “Western” diet. Indeed, at 10 wk, the 12/15-LO KO animals were protected when fed a high-fat diet, but this effect was largely gone by 14 wk. Furthermore, no benefit for transgenic overproduction of 15-LO was seen on the high-fat diet. Human endothelial cell (HUVEC) production of ICAM-1 was increased fivefold by serum from 12/15-LO deficient animals (when compared with wild type) but only when the mice were fed a low-fat chow diet. On a high-fat diet, serum-stimulated production of ICAM-1 was nearly the same. Plasma LXA₄ was much higher in 12/15-LO transgenic animals when fed a chow diet, but on a high-fat diet, plasma LXA₄ was similarly elevated in both transgenic and wild-type animals (1198).

In the most recent study of 12/15-LO KO animals, the mice were fed either high saturated fat (SAT) or high polyunsaturated (PUF) diets. Deletion of 12/15-LO clearly decreased atherosclerosis for both diet groups, but the decrease was greater in PUF-fed group (1507). This phenomenon may have been explained by a substantial reduction in serum cholesterol and triglycerides due to 12/15-KO that was seen only in the PUF-fed animals. 12/15-LO replete Kupffer cells (which are derived from monocytes) appeared to generate a product from a PUF precursor that acted on hepatocytes to upregulate fatty acid synthesis, promote fatty liver, increase VLDL production, and increase plasma cholesterol and triglycerides, explaining at least some of the reduction of atherosclerosis when 12/15-LO was knocked out (1507). However, proinflammatory products of 12/15-LO in artery wall macrophages could have acted directly to promote atherosclerosis as well.

To summarize findings regarding 12/15-LO and atherosclerosis, the major effect of the gene may be protective in the setting of a low-fat diet due to the role of 12/15-LO in lipoxin production. The fact that 5-LO can supply a precursor for lipoxin production may help explain the generally negative results from 5-LO and FLAP deletion studies as well. However, at least early in the course of rapidly progressing atherosclerosis produced by high-fat feeding in hyperlipidemic animals, the pro-inflammatory signaling effects from 12-HETE produced by 12/15-LO seem to predominate. This assessment would suggest that in the human setting, where hyperlipidemia is generally much less severe and plaques develop over decades, one might expect a protective role for 12/15-LO activity. Indeed, this seems to be the case as manifest by a 1.3- to 1.6-fold increase in risk of CAD associated with carrying a single copy of a low-activity 12/15-LO variant in two large epidemiological studies (84). Yet even here, the ambiguity continues since the rare homozygotes were, if anything, protected.

6. Neutrophils are involved in atherosclerosis: at least in early stages

Many of the same receptors and chemokines reviewed in relation to monocyte recruitment and transmigration also mediate neutrophil recruitment into atherosclerotic plaques. CXCR2 is more strongly related to neutrophil recruitment and was strongly atherogenic as was at least one of its ligands, CXCL1 in hyperlipidemic mice (see TABLE 6). In ApoE-deficient mice, neutrophil recruitment to larger arteries early in atherogenesis seemed to depend on CCL5 from platelets being deposited on endothelial cells and then being recognized by CCR5 on neutrophils. A high-fat diet increased neutrophil production in the marrow and mobilization into the bloodstream while also decreasing removal rate from the circulation. Furthermore, neutrophil depletinhibited plaque development in these mice at 4 wk but not at later time points (445).

Earlier studies often lacked the ability to specifically detect neutrophils in plaques but more recent techniques clearly disclose the presence of neutrophils in early plaques of hyperlipidemic mice (1649, 1858). Neutrophils were strongly associated with unstable plaques in coronary arteries in a human autopsy series (1285). Similar evidence was presented for human carotid plaques (827). Neutrophil extracellular traps (NETs) have recently been observed in and on human plaques (1179). Neutrophils recruited to early plaques are found in shoulders near macrophages, but undergo apoptosis and efferocytosis relatively rapidly after they move into plaques (1649). Neutrophils adhering to endothelium release azurocidin which facilitates monocyte adhesion. Furthermore, proteins released from neutrophil granules, such as proteinase 3, directly activate chemokine
production by endothelial cells and appear capable of greatly increasing the affinity of certain chemokines for their receptors on monocytes. Neutrophil cathelicidin (LL37) promotes differentiation of monocytes into more inflammatory macrophages (1846); surprisingly, LL37 also promoted endothelial recovery after injury and limited neointimal hyperplasia (1650). Neutrophils also help promote macrophage activation by secreting TNF-α, IL-8, and IFN-γ as well as myeloperoxidase (444). Neutrophil phagocytosis is triggered by innate cues followed by prodigious release of MPO (myeloperoxidase).

7. Natural killer T-cells and related

Natural killer T-cells (NKT) with an invariant TCR (also referred to as CD1d-restricted NKT) can respond to glycolipids presented on the CD1d receptors of antigen-presenting cells. When thus activated, NKT quickly release copious amounts of TNF-α, IFN-γ, MCP-1, IL-5, and IL-6, with lesser quantities of IL-2 and IL-4 (1821). These cytokines presumably further activate nearby NK, Th1, Th2, macrophages, and endothelial cells.

NK, NKT, γδ T, and CD8+ αβ T cells express a receptor named KLRK1 (killer cell lectin-like receptor, subfamily K, member 1; also known as NKG2D for NK cell lectin-like receptor subfamily K). KLRK1 is a membrane-bound receptor that binds a number of “danger signal” ligands expressed at very low levels on healthy cells but greatly upregulated on infected or injured cells. In humans, KLRK1 ligands include two major histocompatibility complex class I chain-related family members (MICA and MICB) and 5 UL16-binding protein family members (ULBP1–4 and RAET1G). Expression of MICA/B was shown to be clearly increased in human atherosclerotic plaques (on EC and macrophages) with soluble receptors detected in plasma of diabetic subjects. Expression of KLRK1 ligands were markedly increased in hyperlipidemic as well as diabetic mice. KLRK1 KO markedly decreased atherosclerosis by 80% (1989). The KLRK1 receptor and ligand system seems to work similarly to TLR ligation to induce rapid and generous production of inflammatory cytokines including IFN-γ, TNF-α, CXCL1, IL-1β, IL-6, and IL-12 (1118).

8. Mast cells in atherosclerosis

While activated by IgE antibodies which are directed against specific antigens (particularly found on helminths), mast cells are discussed here because they secrete several products that might be rightly classed as innate immune mediators and because they are activated in response to essentially any antigen-bound IgE regardless of the antigen. Th2 cells are required for the production of such IgE (1). Their IgE receptor (FceRI) is analogous to the activating, ITAM-containing Fcy receptors on macrophages and also utilize γ-chains that mediate inflammatory signaling. When activated they released preformed histamine stored in their characteristic granules but also CCL3, IL-4, IL-5, IL-6, IL-13, PAF, TNF-α, IFN-γ, renin, PGD2, LTC4, LTD4, LTE4, and GM-CSF.

Histamine appears to be proatherogenic. In older studies of immune injury, antihistamine administration decreased atherosclerosis in hyperlipidemic rabbits (1). More convincingly, KO of histidine decarboxylase (HDC), the rate-limiting enzyme for histamine synthesis, was protective. Nuclear NF-κB, inflammatory cytokines, and MMPs were reduced in HDC-null mice despite higher cholesterol levels (1900). Histamine receptor H1 (HRH1)-deficient mice had reduced atherosclerosis together with less vascular permeability to LDL. Furthermore, a 40% reduction in atherosclerosis was seen with an H1 antagonist (mepyramine) in this study. Endothelial HRH1 seems to be central to this effect as transplant of HRH1−/− bone marrow did not affect atherosclerosis (1515). In contrast, no effect on atherosclerosis was seen with HRH2 KO or an H2 antagonist (ranitidine) (1515).

Kit is a transmembrane tyrosine kinase receptor for stem cell factor (SCF), a growth factor required for mast cell differentiation. The name Kit seems not to be an abbreviation and its naming is unexplained (138). KitW-sh/W-sh mice have no mast cells. They had reduced atherosclerosis but apparently because of combined deficiency of IL-6 and IFN-γ, which are normally secreted by mast cells (1709). Deficiency of mast cell chymase decreases aneurysm formation but apparently not atherosclerosis (1710) despite elaborate hypotheses regarding potential contributions of chymase to atherogenesis (943).

GM-CSF is an important growth factor secreted by mast cells. GM-CSF is a major stimulus for neutrophil development and also appears to support DC in atherosclerotic lesions. GM-CSF clearly promoted intimal DC proliferation in early lesions, but entry of monocytes was not affected (2112). GM-CSF can also be secreted together with M-CSF by activated EC. Even though GM-CSF with IL-4 can promote an M2-type macrophage phenotype, KO of GM-CSF reduced atherosclerosis in LDLR-null mice (918).
brane-bound IgM or IgD accompanied by heterodimers of ple copies of CD3. The BCR is composed of a plasma membrane adaptors for the TCR complex include invariant adaptor proteins found in the TCR complex. Tyrosine kinases which then phosphorylate ITAM sequences into its extracellular antigen-binding site, but its cytoplasmic tail contains no ITAM or ITIM and is not covalently modified after antigen binding. Instead, TCR activation is driven by Src-family tyrosine kinases to the TCR complex, bound to their cytoplasmic tails. CD4+ T-cells include Th1, Th2, Th17, and T-regulatory cells (Treg). CD8+ T-cells are cytotoxic T lymphocytes (CTL).

Th (CD4+) cells generally respond to extracellular antigens such as extracellular bacteria that have been phagocytosed by macrophages (but can also respond to cytoplasmic proteins taken up by autophagy), while CTL (CD8+) typically respond to intracellular antigens (such as those generated in virus-infected cells), often leading to killing of the infected cell. Activation after TCR recognition of antigen is contingent on the expression level of costimulatory and coinhibitory receptors on the presenting cell.

The TCR incorporates variable immunoglobulin-like sequences into its extracellular antigen-binding site, but its cytoplasmic tail contains no ITAM or ITIM and is not covalently modified after antigen binding. Instead, TCR antigen binding activates CD4 or CD8-bound Src-family tyrosine kinases which then phosphorylate ITAM sequences in invariant adaptor proteins found in the TCR complex. Adaptors for the TCR complex include ζ-chains and multiple copies of CD3. The BCR is composed of a plasma membrane-bound IgM or IgD accompanied by heterodimers of the invariant transmembrane adapter proteins Igα and Igβ, both of which contain ITAM sequences. Similar to the TCR, when the IgM of the BCR binds a foreign protein, a membrane-bound Src-family tyrosine kinase is activated to phosphorylate tyrosines in the ITAM sequences of Igα and Igβ. IgM and IgD of the BCR and the TCR variable sequences are synthesized during cell differentiation by a process of random shuffling of specific DNA sequences. Once mature, these rearrangements are preserved in selected clones of cells (836, 1053). One particular clone of T- or B-cells expresses only one specific variable sequence on its TCR or BCR. Proliferation of a particular clone after antigen recognition and activation constitutes immunologic memory.

The TCR recognizes only relatively small peptides bound to MHC receptors. In contrast, the BCR can recognize and bind more complex shapes or sequences on the surface of foreign cells or proteins in their native state (without the need for prior digestion and presentation). When the B-cell binds such a native, foreign protein, it begins activation followed by phagocytosis and endosomal digestion of the invader or protein bound to its BCR. Foreign peptides derived from this process can then be loaded onto the B-cell’s MHCII receptor and presented to CD4+ Th cells. Th cells previously activated by the same antigen presented to their TCR by nearby DC will then present sufficient costimulating receptors and secrete activating cytokines to promote efficient proliferation of the B-cells. The antibodies eventually produced by the active clone of B-cells will match the antigenic specificity of the original BCR IgM or IgD rather than the antigen (peptide) presented by the MHCII receptor. Absence of activating costimulatory signaling blocks activation and may signal for apoptosis as may the binding of coinhibitory receptors (122, 646).

After antigen recognition, IL-2 becomes critical for proliferation of activated T cells and further differentiation into various subsets. In chronic inflammation, Th1 and Th2 subsets predominate, with Th1 considered the main Th subtype in both human and mouse atheroma based primarily on observations on predominant cytokines present, such as IFN-γ, the prototypic Th1 cytokine (61, 713). IFN-γ enhances the immune response in part by stimulating an increase in class II MHC receptors on antigen-presenting cells. After IFN-γ binds its JAK/STAT receptor, there is activation of STAT1, STAT1, together with other transcription factors, upregulates expression of the master regulator of MHCII receptors, CIITA (or C2TA, class II transactivator). By acting as a kind of scaffold to assemble necessary transcription factors in a specific order, but without binding DNA itself, CIITA induces transcription of MHCII receptors (1471). KO of CIITA in ApoE-deficient animals has been reported to increase abdominal aortic aneurysms, with excess Th2 activation and metalloproteinase expression, but no effect on atherosclerosis was mentioned (1625). IFN-γ also stimulates the transcription factor T-bet and IL-12 to promote Th1 development. Furthermore, IFN-γ stimulates Th1 cells to become major IFN-γ producers themselves, with IFN-γ promoting classical macrophage activation (resulting in M1 macrophages) and enabling infected macrophages to kill phagocytosed invaders. M1
Macrophage deactivation occurs in response to Treg cytokines TNF-β and IL-10 with downregulation of MHCII receptors. Importantly, ingestion of apoptotic cells (if these targeted cells do not themselves express or contain “danger signals”) also leads to macrophage deactivation and production of TNF-β and IL-10 (286). Macrophages and neutrophils can recognize targets coated by antibody by their Fc receptors, while other receptors recognize complement or lectin-coated invaders. Receptor binding is followed by phagocytosis and killing. Thus macrophages and neutrophils are effectors for both innate and adaptive immunity.

2. Which predominates in atherogenesis: innate or adaptive immunity?

For a number of years it was thought that macrophages were so predominant in plaques that essentially only the innate immune response was relevant to atherogenesis (580). Indeed, many if not most components of the innate immune system are critically involved in atherogenesis (580, 1088). After all, marked reductions in atherosclerosis had been seen after interfering with many aspects of innate immunity, including TLR signaling (TABLE 3); scavenger receptors (to be reviewed below), adhesion molecules and chemokines (TABLE 6); various aspects of cytokine signaling including TNF, IL-1 signaling, MIF, and IFN-γ (TABLE 7); NKT activation by innately recognized glycolipids (TABLE 7); and neutrophil activity (1649, 1933).

The notion that adaptive immunity was unimportant seemed to be supported by a report that deficiency of Rag1 (recombination activator gene 1) or Rag2 in fat-fed ApoE KO mice led to only a very early reduction in atherosclerosis which diminished with time (379, 382). Interestingly, the protective effects of estrogen in ovariectomized mice were lost in Rag2-deficient mice or mice transplanted with Rag2-deficient marrow but could be restored by normal Rag2+/− marrow transplant. No specific subset of B or T lymphocytes could be identified that were responsible for this effect (470).

Rag1 and Rag2 form a tetramer known as V(D)J recombinase with Rag1 making a nick in the DNA at coding/non-coding junctions of variable (V), diversity (D), and joining (J) gene segments of immunoglobulins or corresponding receptors in developing lymphocytes. DNA rearrangements and annealing follow. Rearranged DNA then directs transcription and translation of both mature Ig and T-cell receptors (TCR). Without normal surface expression of Ig or T-cell receptors, developing lymphocytes undergo apoptosis. Rag1 or Rag2 deficiency thus leads to complete absence of both T and B lymphocytes and immunodeficiency. However, knockout of Rag1 or Rag2 would have also eliminated the anti-inflammatory T-regulatory (Treg) cells together with Th cells.

Later, other models clearly showed that suppression of adaptive immunity (particularly Th cells) led to protection from atherosclerosis (e.g., scid/scid mice, CD4 KO, TCRβ subunit KO see TABLE 7). CD8+ CTL seem to have less of a role as CD8 KO had little effect on lesions in ApoE- or LDLR-deficient mice (TABLE 7) (469, 470). Adoptive transfer, transplant, and several knockout studies in mice further demonstrate that both arms of the immune system are major contributors to atherosclerosis (61, 580, 1130, 1933). Indeed, current models of atherosclerosis depict not only a variety of immune cells in the plaque (particularly macrophages and T-cells), but an inflammatory lesion richly endowed with numerous B-cells in adjacent adventitia as well (580).

IL-4 is a key Th2 cytokine that promotes M2 macrophage activation. Effects of genetic manipulation or infusion have been quite mixed. Some studies show some reduction in atherosclerosis with blocked IL-4 signaling, while a more recent study using multiple models (IL-4 KO in ApoE- or LDLR-deficient mice, with or without ANG II infusion, and IL-4 infusion). None of the models showed an effect on atherosclerosis (913). Other Th2 signaling, namely, release...
of IL-5 and IL-33, appears to be more anti-inflammatory and antiatherogenic (see Table 7).

Th17 cells differentiate from naive CD4+ cells when stimulated simultaneously by TGF-β and inflammatory cytokines such as IL-1 or IL-6. Their net contribution to atherosclerosis remains controversial. KO of the IL-17 receptor decreased atherosclerosis while KO of at least one IL-17 isoform (IL-17A) clearly increased atherosclerosis in ApoE-deficient mice (see Table 7).

The role of CTL in atherosclerosis may need to be reassessed based on recent data. Consistent with older evidence that CTL are less important for atherosclerosis, deletion of TAP1 or CD8 in ApoE-deficient mice had no effect on atherosclerosis (932). However, complete absence of CTL or TAPI during development may lead to compensatory increases in Th, NK, or γδT cell responses or absence of a strongly anti-inflammatory CD8+ regulatory CTL (972). CD8+ CTL are said to be the predominant leukocyte in advanced human plaques, constituting up to 50% of such cells (972). In ApoE-deficient mice, antibodies against either the α or β isoform of CD8 decreased atherosclerosis while reconstitution of lymphocyte-deficient mice with CTL increased atherosclerosis. Transfer of CTL deficient in either perforin, granzyme B, or TNF-α, however, resulted in much less of an increase in atherosclerosis compared with transfer of wild-type CTL. In this study, CTL appeared to target macrophage, VSMC, and endothelial resulting in increased indexes of apoptosis, necrosis, several markers of inflammation (such as MCP-1) and increase necrotic core size (972). The authors suggest that in developing plaque, MHCI may present peptides derived from oxLDL (although no MHCI antigenic peptides promoting atherosclerosis have been identified directly) to CTL prompting release of their granules containing perforin and granzyme B, the main cytotoxic molecules utilized by CTL. The resulting necrotic cells could promote sterile inflammation and increased apoptosis of surrounding macrophages and other cells.

E. T-regulatory Cells in Atherosclerosis

1. Evidence for the atheroprotective effects of Treg

Treg cells are critical for normal immune self-tolerance and are generally anti-inflammatory. Overexpression of IL-10, the key anti-inflammatory cytokine secreted by Treg, effectively reduced atherosclerosis in LDLR KO mice (1885). However, Th2, B1a cells, as well as M2 and Mheme macrophages also produce IL-10, so these data do not prove a specific role for Treg. In the first study to provide clear evidence for Treg inhibiting atherosclerosis, ApoE KO mice were initially injected subcutaneously with either saline or ovalbumin in complete Freund's adjuvant. Mice given the ovalbumin developed strong Th1 and IgG responses against ovalbumin. Both of the two groups of mice were then given intraperitoneal infusions of either saline or clones of ovalbumin-specific Treg, which had been expanded in vitro (with each mouse receiving just 1 million cells). Only in the mice previously sensitized to ovalbumin would the adoptively transferred, ovalbumin-specific Treg be expected to proliferate (being stimulated by specific antigen-presenting cells). This group developed ~30% less atherosclerosis than the other three groups including the group that had received the Treg that had not been previously sensitized to ovalbumin. Importantly, the ovalbumin-specific Treg injected into sensitized mice found their way into growing plaques and adjacent adventitia where IL-10 production was greatly increased together with a marked reduction of macrophage and T-cell accumulation. In addition, there was reduced IFN-γ production by lymph T-cells when stimulated by ovalbumin (1126).

In contrast to the above study showing suppression of atherosclerosis by a bolstered Treg response, adoptive transfer of bone marrow that was deficient in Treg into irradiated mice led to increased atherosclerosis (33). The transcription factor Foxp3 is critical for differentiation and maintenance of the Treg phenotype. Artificially inducing a cytotoxic response toward Foxp3-bearing cells (by vaccination with DC transgenically modified to over-express and present Foxp3) decreased Treg and increased atherosclerosis (1852). Multiple additional genetic manipulations that impair Treg activity also promote atherosclerosis (see Table 7). More recently, tolerance to specific human APOB-100-derived peptides was induced in ApoE-deficient mice by subcutaneous delivery of relatively low doses of the peptides subcutaneously without adjuvant. This was achieved by an osmotic minipump implanted for 14 days. This tolerogenic protocol led to increased Treg activity and a reduction in atherosclerotic plaque development as well as arrest of further progression of plaques. Treg from APOB-100 tolerant mice specifically suppressed proliferation of Th1 cells from mice that had been sensitized to the same APOB-100 peptides. Thus tolerogenic Treg suppress cells that actively present the specific antigen recognized by the Treg. Depletion of Treg abrogated the antiatherosclerotic effect of the treatment (751). These studies also identify APOB-100 as a likely target of proatherogenic adaptive immune responses.

Increasing the number and activity of Treg appears to be the main mechanism whereby repeated oral or nasal administration of low to moderate doses of various antigens (induction of mucosal tolerance) leads to a reduction in atherosclerosis. Targets for tolerance induction have included oxLDL (with decreased atherosclerosis after an oral tolerance protocol), malondialdehyde treated LDL (no effect) (1859), HSP60 (reduced atherosclerosis) (1860), HSP65 (reduced atherosclerosis) (1142), and β2-glycoprotein I (reduced atherosclerosis) (606) in addition to the APOB-100 studies
above. In addition to these "self" targets which had been hypothesized to be involved in the atherogenic sensitization of Th1 cells, mucosal tolerance protocols directed against pneumococcus, or even measles virus envelope resulted in reduced atherosclerosis, suggesting there may be multiple, unsuspected potential antigenic targets as well the possibility of nonspecific effects from simply increasing Treg numbers with increased secretion of IL-10 (30, 241). Indeed, the ovalbumin sensitization study reviewed above would suggest that the Treg stimulus need not be directly involved in atherosclerosis.

2. Effects of costimulatory and coinhibitory receptors on Treg and atherosclerosis

The initially confusing results of genetic deletion studies for a number of costimulatory and coinhibitory receptors are harmoniously explained when effects on Treg cells are considered (see Table 7). For example, CD28 is the best-studied activating costimulatory receptor on T-cells. Without CD28 or other costimulation, Th cells become anergic or unresponsive when their TCR binds an antigen-presenting MHCII receptor, even if the antigen is foreign. But CD28 is also required for Treg differentiation. As it turns out, the net effect of CD28 loss was a dramatic reduction in thymic Treg numbers and an increase in atherosclerosis. The same was found for the costimulator ICOS (inducible costimulator) (see Table 7).

T-cell CD28 interacts with B7-1 (CD80) or B7-2 (CD86) found on antigen presenting cells as well as on Th cells. Activation of innate immune responses in antigen presenting cells increases the surface expression of B7-1/2. Whole body knockout of B7-1 and B7-2 decreased atherosclerosis modestly and was accompanied by a reduction of Th1 cells. In contrast, bone marrow transplant of B7-1/2-deficient cells greatly impaired production of Treg with less effect on Th1, resulting in increased atherosclerosis.

Inhibitory cytotoxic T-lymphocyte antigen 4 (CTLA4) receptors on Treg are also important. CTLA4 has much greater affinity for B7 receptors than CD28. When antigen-presenting cells are not strongly activated, the relatively few B7 receptors may be fully occupied by CTLA4, thereby blocking activating signals to Th effector cells by out-competing CD28 receptor binding. Also, the CTLA4 cytoplasmic tail activates protein phosphatase 2A (PP2A), which dephosphorylates and limits PKB activity, thereby supporting Treg differentiation. Recently, feeding active 1,25-dihydroxyvitamin D3 to ApoE-deficient mice was found to decrease atherosclerosis together with a marked increase in Treg cells and increased CTLA4 signaling (1745). Interestingly, CTLA4 deficiency also causes autoimmune disease.

Programmed death ligand 1 (PD-L1) is a major inhibitory ligand expressed on antigen presenting cells, endothelial cells, Treg, and a number of other body cells. The receptor for PD-L1, PD-1, is found on naive CD4+ cells and Treg. PD-1 on Treg seems to be critical for stimulating and maintaining peripheral Treg differentiation when combined with TCR signaling (537). Indeed, TCR signaling upregulates PD-1 expression on all T-cells and BCR stimulates PD-1 expression on B-cells. Thus the potential for inhibition is enhanced at the same time Th-cells and B-cells are stimulated. Binding of PD-1 on these effector cells by PD-L1/2 tends toward inhibition of further inflammatory responses while increasing Treg activity. The cytoplasmic tail of ligated PD-1 activates the phosphatases SHP1/2 and PTPN which effectively block PI3K and PIP3 signaling, respectively. Th1 cell activation through TCR binding is dependent on PI3K activity and its product PIP3, with subsequent PKB and mTOR signaling, all of which are supported and amplified by costimulatory receptors.

If the TCR of a naive T-cell binds the antigen-presenting MHC II of an unstimulated antigen-presenting cell (such as a DC), then the naive T-cell will find PD-L1 on the DC binding to PD-1 on its own cell surface. Without counter-balancing costimulatory signaling, this naive T-cell will undergo either apoptosis or become anergic. If TCR binding and PD-1 ligation is combined with exposure to TGF-β, the cell can differentiate into a Treg (537). PD-L1/2 or PD-1 (221) deletion increased atherosclerosis together with a reduction in Treg cells and an unopposed increase in Th1 activity.

In an important recent study, DC were rendered tolerogenic to APOB-100 by exposing the cells to APOB-100 together with IL-10. Such cells, when administered as a single injection into hyperlipidemic mice, decreased atherosclerosis by 70%. These mice were transgenic for human APOB-100 and LDLR-deficient. In vitro, the tolerogenic DC displayed modestly reduced TNF-α and MCP-1, but had almost no IL-12 production. Furthermore, the APOB-100/IL-10 pulsed DC specifically inhibited CD4+ Th1 cell proliferation and reduced IFN-γ production in response to APOB-100. At the same time, they stimulated differentiation of antigen-specific Treg cells that also suppressed Th1 responses to APOB-100 (755). In contrast, injection of DC pulsed with malondialdehyde-modified LDL (without concomitant IL-10 treatment) actually aggravated atherosclerosis (769).

3. Treg stimulation and differentiation

Due to the studies showing an anti-atherogenic role for Treg as well as increasingly recognized roll for impaired Treg function in autoimmune disease, intense research efforts have led to increased understanding of Treg differentiation and related signaling. The expression of the transcription factor Foxp3 is now considered a defining feature of Treg (537). Treg generally express CD25 (the IL-2Rα chain) as well. There are other Treg subsets, including CD8+ Treg,
but the focus here will be on CD4+/CD25+ Treg cells expressing the transcription factor Foxp3.

Treg can be broadly divided into natural Treg (nTreg), derived from thymus selection during development (mostly completed before birth in humans), and peripheral or induced Treg (iTreg). “Self” antigens are presented without costimulator receptors by thymus cells throughout development, triggering apoptosis in those developing T-cells that have a TCR which strongly binds these antigens. However, a number of developing CD4+ cells with modest binding are selected and differentiate into nTreg. Interestingly, nTreg differentiation in the thymus is dependent on low levels of NF-κB activation and IL-2 production as well as the calcineurin-NFAT pathway. nTreg development is obliterated by CARMA1 deficiency (part of the CBM complex that activates NF-κB in T-cells, reviewed previously) (1225).

Induced differentiation of naive CD4+ into iTreg can be achieved in vitro by mild antigenic exposure (TCR and mild CD28 stimulation) together with TGF-β and IL-2 (potentially induced by internal NF-κB activation or externally supplied). A recently recognized receptor (glycoprotein-A repetitions predominant, GARP) holds and activates surface latent TGF-β and thereby helps perpetuate stable Treg differentiation. GARP is uniquely expressed on Treg (and is dependent on Foxp3) and may aid in positive identification of human Treg (1438).

In vivo, iTreg production can be produced by tolerogenic antigen administration (often by oral administration or systemic administration in water without adjuvants). This type of antigenic exposure is presumably not accompanied by innate activation and thus should allow antigen presentation without costimulatory receptor activation. In contrast, at high levels, stimulation of both the TCR and the activating costimulatory receptor CD28, naive CD4+ resist other signals to differentiate into Treg and instead become effector cells capable of secreting TNF-α, IFN-γ, IL-9, and IL-17. These changes are mediated by high level activation of NF-κB and subsequent production of TNF-α and IFN-γ (1225) as well as increased PI3Kδ activation through CD28 (591, 1585).

Foxp3 is the master transcription factor leading to Treg differentiation. Multiple different transcription factors control Foxp3 expression by binding to four separate sites in the 5’ end of Foxp3 (1199). On the first site NFAT, NF-κB, AP-1, Stat1, Foxo1/3a, Runx, and STAT5 (from IL-2R JAK/STAT signaling) all bind. At the next site, NFAT and SMAD2/3/4 (from TGF-β signaling) bind. Further downstream at the third site, NF-κB, CREB, ATF, Foxo1/3a, Runx, STAT5, and remarkably, Foxp3 itself bind. Foxp3 acting as its own positive DNA regulating factor suggests a means for stable, continued expression. Finally, NF-κB binds the fourth site. Elucidation of this remarkable and elegant complexity, with multiple “AND switches” provides an explanation for the observed inhibition of Treg differentiation by PI3K and PKB. PKB phosphorylates Foxo1 and Foxo3a, thereby excluding these required transcription factors from the nucleus. Activation of PI3Kδ followed by PKB through the combined signaling of the TCR and CD28 is one way that typical, pro-inflammatory Th1 activation prevents transcription of Foxp3 and excessive differentiation of CD4+ cells into Treg (176). Understanding of Treg differentiation to this degree has been strongly motivated by the hopes of developing Treg-based therapies for autoimmune diseases such as multiple sclerosis, type I diabetes, rheumatoid arthritis, and others, but may be relevant to atherosclerosis as well.

How Foxp3 transforms CD4+ Th cells into Treg and how Treg exert their inhibitory effects on Th (and other effector cells) remain major research questions. The field remains in flux and full of complexity, so only a few, probably over-simplified answers to these two questions will be attempted here. Of great importance is the balance between the activities of PI3Kδ-PKB versus the E3 ubiquitin ligase Cbl-b (Casitas B-lineage lymphoma sequence B). Increased PLCγ and particularly PKCθ activity (followed by CARMA, Bcl10, MALT1 activation of NF-κB) also promote Th1 activation. Finally, the particular combination of transcription factors found together with NFAT influences activation.

Expression of Cbl-b is directly or indirectly increased by Foxp3, and absence of Cbl-b causes severe Treg deficiency, excess Th activity, and autoimmune disease (279). Targets of Cbl-b monoubiquitination include PKCθ, PLCγ, Src and Syk family kinases (such as ZAP70), the regulatory p85 subunits of PI3K (the p85 regulatory subunits are shared by class Ia PI3Ks), and the TCR complex itself (by ubiquitinating ZAP70 and CD3 subunits). The result of monoubiquitination by Cbl-b is inactivation without degradation (for p85) or targeting for endosomal delivery and lysosomal (not proteosomal) proteolysis. In opposition to Cbl-b is PKCθ, which, when activated by CD28 signaling, binds to and phosphorylates Cbl-b, apparently targeting it for ubiquitination and degradation (1559). Thus high PKCθ impairs Cbl-b while high Cbl-b blocks Th cell activation. The outcome of this tug-of-war seems largely determined by PI3Kδ activity and subsequent PKB activation, with both controlled by the balance of costimulatory signaling through CD28 versus coinhibitory signaling by CTLA4 and PD-1 (537, 1559, 1585). Foxp3 plays a key role in this balance.

When Foxp3 expression rises, nuclear Foxp3 forms a physical complex with NFAT and subverts NFAT signaling from Th activation toward the anti-inflammatory Treg phenotype (1146, 1163, 1531, 1579). Production of Th1 and...
Th2 cytokines is repressed while TGF-β and IL-10 are induced. Although IL-2 production is suppressed, CD25 (the IL-2Rα subunit) is increased along with STAT5 activation, facilitating Treg survival and proliferation. Expression of the coinhibitory receptor CTLA4 is upregulated as is Cbl-b. The micro-RNA miR-155 is increased which suppressed SOCS1 and thereby increases STAT5 signaling (1083). STAT5 signaling supports Foxp3 production as well as many other functions including hematopoietic cell proliferation.

Multiple mechanisms account for suppression of effector cells by Treg. Treg bound by its TCR to the MHCII receptor of an antigen presenting cell seems to result in a downregulation of B71/2 expression by the antigen-presenting cell. This may be mediated, in part, through the CTLA4 receptor on Treg. Thus antigen-presenting cells exposed to Treg are suppressed in their ability to activate Th and CTL. Treg express cell surface PD-L1 as well as PD-1 and can thereby directly inhibit Th cells expressing PD-1 (1696). In contrast, expression of FAS and decrease effector cell activation by hydrolysis of extracellular ATP by cell surface CD73 and CD39.

**F. Humoral Immunity and B Cells in Atherosclerosis**

1. **Natural IgM antibodies**

The role of natural antibodies in atherogenesis will be further examined here, recognizing that these antibodies are considered part of the innate immune system since they are coded in germ cells and expressed without the need for antigen presentation. Despite this innate production, moderately aggressive immunization against modified LDL or certain other antigens (for example, with coadministration of adjuvant) increases production of natural IgM antibodies by B1a cells, complicating the interpretation of many earlier studies of immunization with oxLDL. Most of these IgM antibodies are directed at phosphocholine groups on oxidized phospholipids or bacterial cell membranes (59, 728). Genetic ablation of genes required for B1a cells to produce IgM clearly increases atherosclerosis, verifying the protective nature of these antibodies (see **TABLE 7**). Passive immunization studies also demonstrate that these IgM antibodies, which accumulate in plaques, were indeed protective (59, 970). Natural IgM antibodies seem to coat modified lipoproteins (such as oxLDL) and decrease macrophage uptake, possibly decreasing foam cell formation. In addition, efferocytosis by macrophages is promoted when natural IgM opsonize apoptotic cells (which have phosphocholine antigens exposed as oxidized phospholipids) (971). Other circumstantial evidence suggests natural IgM are protective (554). B1a cells can also secrete anti-inflammatory IL-10 (971).

2. **The atherogenic effect of IgG appears to depend on the target**

Unlike production of natural antibodies, humoral immunity directed against most antigens consists primarily of IgG produced by mature B2 B cells activated by adaptive immune responses. When directed against various “self” targets, these adaptive immune responses are generally proinflammatory and atherogenic (603). In contrast, immunization with certain aldehyde (MDA) modified APOB-100 peptides (including MDA-p45 and MDA-p74) led to a marked increase in Th2-specific IgG1 antibodies directed against these modified peptides and decreased atherosclerosis in ApoE-deficient mice (545). Furthermore, administration of human recombinant antibodies against these APOB-100 peptides also decreased atherosclerosis in hyperlipidemic mice, possibly associated with facilitated removal of oxidized LDL or decreasing associated inflammation (1554). While much of the immunization benefit in mice may have been due to Treg stimulation (1960), the potential for the recombinant antibodies to protect humans is currently being investigated in a clinical trial [A Study to Evaluate the Safety, Tolerability, and Activity of Intravenous MLDL1278A in Patients on Standard-of-Care Therapy for Stable Atherosclerotic Cardiovascular Disease (GLACIER: Goal of Oxidized LDL and Activated Macrophage Inhibition by Exposure to a Recombinant Antibody); www.clinicaltrials.gov].

Several additional studies address the role of specific IgG. Intraperitoneal infusion of IgG from mice immunized against HSP65 (which cross reacts with HSP60) led to increased atherosclerosis (604). Infusion of goat anti-human apolipoprotein A1 (APOA1, or A-I) increased atherosclerosis compared with control goat IgG in chow-fed ApoE-deficient mice (1227). On the other hand, passive immunization of LDLR KO mice with a monoclonal mouse IgG2b antibody directed against electronegative LDL decreased atherosclerosis by 77% along with reduced circulating electronegative LDL (663).

Results regarding immune complexes with oxLDL, primarily containing IgG1 and IgG3, further complicate the picture. In one study, oxLDL-IgG immune complexes deposited on cultured human endothelial cells promoted adherence and activation of monocytes (1269). In macrophages, oxLDL immune complexes are taken up mainly by the FcYRI receptor while scavenger receptors such as CD36 mediate uptake of free oxLDL. These oxLDL immune complexes are reported to promote greater foam cell formation,
release of active secretory acid sphingomyelinase, prolonged activation of lysosomal acid sphingomyelinase, and release of more cytokines compared with oxLDL alone. Yet oxLDL-IgG complexes also promote greater macrophage survival with less ROS production, mediated by intracellular FcγRII signaling including Akt activation (36, 704, 1811). The ultimate effect of oxLDL-IgG complexes on atherosclerosis remains unresolved, however. In human studies, there is a report of a positive association between oxLDL immune complexes and coronary calcium in one study (1071) but no association with new onset MI in another cohort (though an increased risk of MI in those with greater MDA-modified LDL-immune complexes was reported) (1074). In another study, greater progression of diabetic retinopathy and nephropathy were seen among patients with increased oxLDL immune complexes (1072, 1073).

Utilizing a very different approach, transgenic mice expressing human APOB-100 were immunized with human oxLDL followed by generation of T-cell hybridomas. The investigators were surprised to find that none of the hybridomas recognized oxLDL epitopes, but only native LDL or intact APOB-100 peptide. Nevertheless, the sensitized Th1 cells could apparently license mature B-cells to produce a much wider array of antibodies against oxLDL as well as native LDL. Inhibition of Th1 cells sensitized to LDL with an antibody specific to the sensitized T-cell receptor decreased atherosclerosis by 65% in APOB-100 expressing LDLR−/− mice, suggesting but not proving a proatherogenic role for anti-LDL IgG or anti-oxLDL IgG (756). At the very least, these results clearly contrast with the protection seen in studies using immunization protocols that actually ended up promoting production of anti-oxLDL IgM natural antibodies. Possibly related to this finding, a proteolytic fragment of native APOB-100 present in human atherosclerotic plaques was recently identified that could activate macrophages and may lead to activation of both innate and adaptive immune responses directed against APOB-100 or a naturally derived proteolytic fragment (897).

With regard to immunization generally, tolerance protocols must not be confused with immunization. In mice, tolerance to an antigen is developed by repeated oral or nasal administration of a relatively low to moderate dose of the antigen. Similar tolerance is the goal of “allergy shots” given to humans as slowly increasing doses of a specific antigen without combined adjuvant. In contrast, human immunization programs with oral polio vaccines or nasal administration of flu vaccine generally utilize much higher doses of antigen, often combined with an adjuvant. Adjuvant is composed of one or more substances that stimulate innate immune responses to ensure that a stimulatory adaptive response will be directed against the simultaneously administered antigen. Mucosal tolerance protocols against specific antigens, rather than inducing specific IgG antibodies, are intended to reduce adaptive (and humoral IgG) responses and enhance iTreg activity, often resulting in reduced inflammation and atherosclerosis.

The example of HSP60 is illustrative. HSP60 expression on the surface of endothelial cells may be atherogenic as suggested by the following evidence. T-cells sensitized to HSP60 and anti-HSP60 antibodies were found in early and advanced human plaques. Plasma titers of anti-HSP60 antibodies were elevated in CAD patients, and these antibodies cross-reacted with Cytomegalovirus HSP65 and were cytotoxic to endothelial cells (114). Animals given oral or nasal desensitizing doses of HSP60 or HSP65 prior to induction of hyperlipidemia showed an increase in Treg cells directed against those antigens and a shift to fewer Th1 cells relative to Th2 cells with increased IL-10 production and greater self-tolerance or anergy toward HSP60. Reductions in atherosclerosis of 50% to as much as 80% were reported (1142, 1861). Whether such desensitization therapy may be effective to prevent progression of human atherosclerosis is unknown. Nevertheless, the results illustrate the principal that adaptive (including humoral) immunity directed against “self” has the potential to be pro-atherogenic, while desensitization may be protective.

Attempts to address the role of humoral immunity have also been made by depleting or replenishing B2 cells. However, B-cells also actively present antigens and can stimulate both T-cells and DC. Also, B-cells actively secrete TNF-α (28, 190). Thus B-cell depletion studies cannot directly address the issue of the role of IgG in atherosclerosis. They are, nevertheless, illustrative of the importance of adaptive immune responses generally. Administration of a monoclonal antibody directed against CD20 (paradoxically also known as B1, a surface marker unique to B2-cells) caused selective depletion of only mature B2-cells and clearly reduced atherosclerosis in high-fat and chow-fed ApoE-deficient mice as well as LDLR KO mice (28). Besides profound reductions in total IgG as well as anti-oxLDL IgG in these studies, depletion of mature B2-cells led to reduced IFN-γ secretion from T-cells and enhanced production of IL-17. Importantly, blocking IL-17 with a neutralizing antibody completely reversed the protective effect of B-cell depletion, without a change in the number of B-cells or anti-oxLDL antibodies (28). Thus effects of mature B-cell depletion on T-cell or DC activation and resulting cytokine milieu may be more important than effects on antibody production in this study. Such an interpretation is consistent with another study that confirmed reduction of atherosclerosis using antibodies against CD20 to deplete B2 B cells and further showed a benefit in established lesions. In addition, a 72% increase in atherosclerosis was seen with adoptive transfer of B2 B cells into ApoE KO mice deficient in B, T, NK and NK cells (ApoE−/− Rag2−/− γc−/− triple KO mice), but a 300% increase occurred with transfer into ApoE KO mice.
deficient only in B cells due to absence of IgM chains (μMT−/−). This study showed B2 cells can increase atherosclerosis in the absence of T cells, but that a much greater increase occurs when T cells are present (969).

Deletion of the BAFF receptor (BAFFR), necessary for B2 development, was also recently shown to be protective in at least two studies (968, 1528). Like the anti-CD20 antibody infusion studies, BAFFR deficiency resulted in marked selective reduction of B2 cells with little effect on B1a cells. However, as noted above, these data cannot resolve the issue of IgG secretion separately from other B-cell activities.

At least some additional functions of B-cells remain unexplained with apparently protective effects from mature B-cells in certain contexts. Id3 (inhibitor of DNA binding 3, also known as inhibitor of differentiation 3) is a protein than can form heterodimers with various transcription factors and inhibit their binding to DNA. In B-cells Id3 apparently limits proliferation and Id3 mutations are frequently found in Burkitt’s lymphoma. Deletion of Id3 resulted in impaired homing of B-cells to the aortic adventitia, an increase in macrophage content of plaques, and increased atherosclerosis (437). This effect was not due to a reduction in B1a function or lower IgM. The mechanism of protection from these adventitial B-cells remains to be explained.

G. Redundancy in Leukocyte Signaling

Numerous examples of redundancy for macrophage and lymphocyte signaling are provided in TABLES 6 AND 7. Redundancy potentially dilutes the effect of any single gene manipulation over time. An example for CCR2 (the receptor for MCP-1) was given in the introduction. In another study, there was a clearly protective effect of heterozygous CX3CR1 deficiency for early atherosclerosis but none for more advanced lesions (1871). In contrast, for CX3CR1 (the fractalkine receptor), heterozygous deficiency resulted in as great a reduction in atherosclerosis as complete deficiency, suggesting a sort of redundancy even in the number of alleles (344). Indeed, redundancy is a recognized characteristic of cytokines generally (1). With few exceptions, functions of a given initiating pathway are only partially, not completely dependent on any one gene. As a result, effects on atherogenesis from disrupting a single gene are generally modest. The potential for intervention on multiple chemokine pathways is illustrated by KO of both CCL2 and CX3CR1, together with blocking CCR5 signaling which almost entirely abrogated atherosclerosis in ApoE-deficient mice (345). However, in this model monocyte mobilization was severely impaired and the reduction in atherosclerosis was directly proportional to the reduction in blood monocyte counts. These mice might therefore be expected to be immunodeficient as well.

Another important example of redundancy is an alternate mechanism besides TLR to activate innate responses. Such alternatives include RAGE and LOX-1 previously reviewed. In addition, hyperlipidemia, diabetes, infection, and injury can stimulate expression of various ligands of the KLRK1 receptor on endothelial cells and macrophages, among other cells. Activation of the KLRK1 receptor causes vigorous production of cytokines by NK, NKT, and other cells while KO of KLRK1 markedly reduced atherosclerosis as noted previously (1118, 1989).

Redundant systems generally govern functions that are vital for survival. In the case of leukocyte transmigration and activation, any intervention must be weighed against the threat of severe infections due to immunodeficiency as well as increased risk of cancer. Interventions that do not impair essential pathways but which bolster natural defenses (as in enhancing natural cellular antioxidant responses by various biologics, supplying various cofactors such as lipoic acid, or modestly increasing H2S availability) or which bring risk factor levels to a more physiological range (such as control of hyperlipidemia or reduction in blood pressure) will likely have more success than efforts to impact a single genetic element of inflammatory signaling. Nevertheless, creative application of newly developed anti-inflammatory agents in a limited or focused setting may well have major beneficial effects.

VIII. EFFECTS OF SELECTED RISK FACTORS ON INITIATION OF ATHEROSCLEROSIS

A. Aging

Age is often cited as the most powerful atherosclerosis risk factor. Accumulating evidence suggests the underlying mechanism involves much more than merely longer exposure to other risk factors. One candidate may be increasing fibronectin, which was shown to accumulate in the arteries of aging rats along with collagen (1905). Both 12-HETE and ANG II can stimulate VSMC to produce fibronectin (1034). It is conceivable that increasing exposure to fibronectin with age could promote an inflammatory phenotype in endothelial cells and enhance susceptibility to atherosclerosis with age.

In a series of investigations from one laboratory, aging of ApoE-deficient mice led to depletion of reparative, bone marrow-derived, vascular progenitor cells. Emergence of atherosclerosis correlated with an empirically derived gene expression pattern suggesting depletion of these reparative cells (884). Depletion of vascular progenitor cells capable of inhibiting atherosclerosis appeared more rapid with prolonged exposure to other risk factors, including hyperlipidemia (1460). This group has begun to identify the subfraction of hematopoietic stem cells that seems to be responsible for the reparative effect and to develop specialized culture conditions (incubation with IL-3, IL-6, and stem cell factor
Mitochondrial damage in the aorta was increased in ApoE-deficient mice compared with normalolipidemic mice. Both the absolute degree of damage and the excess mitochondrial damage seen in hyperlipidemic mice were greatly exaggerated by exposure to second-hand cigarette smoke (922). Hemizygous SOD2 deficiency in ApoE−/− mice increased mitochondrial ROS production upon exposure to TNF-α (724). Incubation of human endothelial cells with high glucose promotes mitochondrial ROS production and mitochondrial damage (815) and high fatty acid levels in diabetes is also likely to contribute to mitochondrial dysfunction (2000). Excess intracellular calcium levels associated with excessive aldosterone in the presence of relatively high salt intake may also contribute to mitochondrial ROS production (2116). Higher ROS may theoretically shift the balance of tyrosine kinases and phosphatases toward the inflammation-activating tyrosine kinases (868).

In many animal studies, modest caloric restriction with reduced insulin signaling increases life span and is associated with improved mitochondrial biogenesis as well as greater removal of defunct or impaired mitochondria by autophagy (or mitophagy) (695). In 18 humans attempting to follow a calorically restricted but nutrient-rich diet, carotid atherosclerosis appeared to be reduced compared with 18 controls (532). The apparent benefit of caloric restriction, however, may be most relevant to relatively overfed animals (or humans) as recent data suggest no survival benefit for caloric restriction in already lean monkeys (1160).

2. Cell senescence: in part aggravated by excess ROS and TNF-α signaling

While still a controversial topic, cell senescence associated with multiple cell divisions may be relevant. After several replications, porcine endothelial cells showed increased NF-κB activity, increased oxidative stress, reduction of several antioxidant enzymes, and increased p53 and apoptosis (993). Aging arterial VSMC tend to be modulated toward the secretory phenotype (1903). In general, aging appears to lead to marked increases in TNF-α signaling (particularly from membrane-bound TNF-α), NF-κB activation, and ROS production in endothelial as well as other cells of the arterial wall (1538, 1834). Even aging skin demonstrates a gene expression pattern characterized by increased NF-κB signaling (14). One of the major contributors to the increase in NF-κB signaling with age may be accumulation of fibronectin (as documented especially in hypertensive rats) (826, 976). Fibronectin might be expected to accumulate particularly in vascular areas hemodynamically prone to atherosclerosis. As noted above, high glucose, as seen in diabetes, promotes increased ROS and mitochondrial dysfunction. Endothelial cells from Zucker diabetic rats were strongly prone to premature senescence with evidence of marked improvement after administration of the peroxynitrite scavenger ebselen (210). In general, evidence that endothelial senescence may contribute to vascular disease is
accumulating, but its relevance to human disease has yet to be demonstrated (488).

The above considerations regarding age-related changes in the vasculature led to the hypothesis that there may be greater susceptibility to atherosclerosis initiation, promotion, and progression in older versus younger arteries exposed to the same standard risk factors such as dyslipidemia and hypertension. Recent evidence suggests this may indeed be the case.

In LDLR-null mice, hypercholesterolemia is not severe and atherosclerosis does not progress appreciably until after institution of a western-style diet. When such a diet was initiated at different ages (3- and 12-mo-old mice), the older mice experienced much more rapidly progressing atherosclerosis despite the same lipid levels. The older mice failed to upregulate antioxidant enzymes as efficiently, although this failure was improved by the PPARγ agonist rosiglitazone (341). Older mice showed increased ROS production stimulated by TNF-α stimulation. When carotid arteries from 2 and 18 wk normolipemic mice were transplanted into ApoE-deficient mice, the older arteries developed atherosclerosis about twice as rapidly but, in addition, showed much greater intraplaque hemorrhage and buried fibrous caps (suggestive of rupture and repair). Importantly, there was a fivefold increase in the expression of TNFR1 in the aged arteries. All these age-related differences were eliminated in carotid segments transplanted from TNFR1−/− mice (2076). In the same paper, a variant of TNFR1 was reported to be associated with CAD together with a significant interaction with age in a human population. The “at risk” TNFR1 allele was associated with a more rapid increase in the extent of CAD in aging subjects (2076). Age-relevant changes in the artery wall, particularly accumulation of AGEs and oxidized or otherwise modified LDL, may be a trigger of TNFR signaling and increased ROS production with impaired sirtuin activity (1170, 2071).

Low-level superoxide production appears constitutive in normal human arteries but is increased in human atherosclerotic lesions, both in the diseased vascular wall and in plaque macrophages (1658). In nondiseased human saphenous veins, NOX superoxide production increased with the number of standard CAD risk factors, particularly hypercholesterolemia and diabetes (683).

3. Might manipulation of proteosomal activity provide a benefit?

Atherosclerotic carotid plaques from elderly subjects showed more evidence of ubiquitination and proteosomal activity than plaques from younger carotid endarterectomy patients (1139). Several proinflammatory pathways are activated by proteosomal activity. Proteosomal degradation of IκB and TRAF3 both promote NF-κB signaling. The potent transcription factor Nrf2 which upregulates many cellular antioxidant systems and the rate-limiting enzyme for tetrahydrobiopterin synthesis (required by eNOS) are also targeted for degradation by the proteosome. While these pathways might suggest a benefit for general proteosomal inhibition, the notion must be balanced by other considerations. Increased oxidative stress seen in aging can result in protein modification and misfolding, with targeted proteosomal degradation of the misfolded proteins (through multiple proteosome subtypes). Inadequate proteosomal activity in the face of excess unfolded or otherwise damaged proteins leads to increased ER stress and inflammation (758, 949, 1213). Aggregated, ubiquitinated proteins that are too large for proteosomes are targeted for “selective autophagy,” thought to be a protective process (1964).

Mild to moderate oxidative stress normally upregulates proteosomal activity while severe oxidative stress is inhibitory. Conversely, mild to moderate proteosomal activity decreases oxidative stress while high activity is said to increase ROS production (758). Some evidence for anti-inflammatory effects of proteosomal inhibition early in atherosclerosis has been presented, but others find increased endothelial activation and intimal thickening with proteosome inhibition even at this stage (758).

Despite the conflicting evidence, a great deal of effort has been expended to examine pharmacological effects of proteosome inhibitors (originally developed as anti-tumor agents). Pharmacological proteosome inhibition blocked VSMC proliferation after arterial injury. Nevertheless, use of the proteosome inhibitor bortezomib clearly made advanced plaques more unstable (758). The bulk of evidence suggests relatively impaired proteosomal activity and inadequate removal of damaged proteins in advanced human plaques which likely contributes to inflammation, a situation that would only be worsened by proteosomal inhibition (758, 1964).

4. New insights from rare laminopathies

Certain rare aging syndromes caused by mutations in the LMNA (lamin A/C) gene have been associated with premature atherosclerosis (35, 1474). Markedly different phenotypes arise depending on where mutations occur in the LMNA gene. In Dunnigan-type familial partial lipodystrophy (caused by R482Q, R482W, R482L, or R582H mutations) and atypical Werner’s syndrome (A57P, R133L, and L140R), relatively severe and early onset of metabolic syndrome and insulin resistance results in sixfold or higher CAD risk, with higher relative risks in women (35, 1474).

Hutchinson-Gilford progeria syndrome leads to devastating MI, stroke, valvular disease, and cardiomyopathy (among many other abnormalities), without attending risk factors or hypercholesterolemia. MI is common even by age 14, which is the median life span (35). This form of progeria is generally due to de novo mutations in codon 608 of
the intima. The mutation adds a cryptic splice site between exons 11 and 12, resulting in a prelamin A with a 50-bp deletion. This defective prelamin A is then farnesylated normally, but the subsequent required step of proteolytic processing with removal of the farnesylated end does not occur, and the abnormal farnesylated product (called progerin) accumulates in the nuclear envelope causing marked disruption of the normal nuclear lamina, distortion in the shape of the nucleus, and altered function of the nuclear envelop and associated interactions with intermediate fibers and chromatin. There is impaired DNA repair, activation of cyclin-dependent kinase inhibitors with cell cycle arrest, impaired mitosis, meiosis, and gene transcription, and rapid cellular senescence (254). Progerin also appears to disrupt not only nuclear interactions with intermediate fibers but also with actin-based microfilaments and tubulin-based microtubules, resulting in general cell fragility, a feature also seen in LNMA-deficient mice and apparently contributing to the cardiomyopathy seen in this syndrome (254). Careful examination of the coronary arteries in humans with this syndrome show that there are atherosclerotic lesions with cholesterol crystals and activated macrophages, but the degree of lipid accumulation is less and there is more fibrous tissue and calcium in the intima compared with usual plaques. There was also a unique adventitial thickening with pronounced fibrosis that may have contributed to the stenosis (942, 1344). Whether impaired endothelial repair leads to this remarkably premature atherosclerosis or some other mechanism is the underlying cause remains unknown. Importantly, progerin was found to be present in plaques from older individuals who did not have progeria, presumably due to variable degrees of alternative splicing, raising the possibility that progerin may contribute to atherogenesis associated with aging generally (1344).

### B. Hypertension

Pressure and flow are the first risk factors to which the endothelium is exposed. Blood pressure in the arterial range is an essential requirement for the development of atherosclerosis. Venous atherosclerosis does not occur even in patients with homozygous familial hypercholesterolemia (226). The relatively rapid progression of atherosclerosis in saphenous veins in coronary artery bypass suggests there is nothing uniquely resistant about veins themselves. Normally, the pulmonary arterial circulation with its systolic pressures of 12–22 mmHg is another entirely protected site. Nevertheless, with pulmonary hypertension, atherosclerotic plaques are commonly seen (620). While other mechanisms leading to altered permeability cannot be excluded, including pressure- or stretch-induced activation of the proinflammatory protein BMP2 (359), the major mechanism whereby higher blood pressure promotes atherosclerosis appears to be through increased pressure-driven convection of LDL and other lipoproteins into the intima (1713). LDL accumulation in the intima of pressurized rabbit aorta increased 44-fold when pressure was raised from 70 to 160 mmHg (362). A substantial portion of the increased LDL transport, especially at higher pressures, may also be due to a stretch-induced increase in permeability of the vessel (1203). Approximately 90% of the convection of LDL into the subendothelial space is reportedly through gaps created between mitotic endothelial cells in areas of low shear stress with only 10% entering by way of transcellular vesicular traffic (248). A thinner glycocalyx, also associated with atherosclerosis-prone areas with low shear stress, also appears to contribute to greater LDL permeability (1844).

Many of the alterations in endothelial function related to shear stress may also be activated by stretch (1489). In addition, VSMC become activated to produce more superoxide in response to stretch (307, 998, 999), with activation of TGF-α, EGFR, and NF-κB pathways (1003). Stretch of isolated carotid arteries, equivalent to 180 mmHg, activated endothelial integrin/focal adhesion signaling, followed by assembly of a complex on the plasma membrane consisting of paxillin and activated ILK-1 with co-localization and activation of the Rac1-GEF, βPIX (PAK-interacting exchange factor β). Rac1 and NOX activation with increased superoxide production ensued (1868). In another study, exposure of carotid arteries to high pressure resulted in activation of NF-κB followed by MCP-1 expression and enhanced monocyte adherence (1489).

### C. Exercise

Even modest exercise serves as a potent stimulus to improve endothelial function as measured by vasodilation in response to acetylcholine, particularly in older animals and humans (415, 457, 1580). Exercise increases eNOS expression in animals and reduces restenosis induced by catheter injury (823, 1979). Exercise stimulates phosphorylation of eNOS by PKB (2078). Aged mice had much higher levels of nitrotyrosine (a marker of chronic oxidative stress) in their aortas compared with younger mice, and this was markedly reduced by modest amounts of voluntary running on an exercise wheel (457). **Figure 11** illustrates several of the mechanisms thought to mediate these benefits. Exercise acutely increases flow, and hence the shear-stress experienced by endothelial cells in many vascular beds, not just the exercising limb. Furthermore, the NO released alters transcription of eNOS and leads to increased expression of eNOS protein. Thus inhibition of eNOS activity abrogates the exercise-induced increase in eNOS protein expression (1335).

Recently, exercise was found to increase telomerase activity in mice. Telomerase then synergized with eNOS to confer impressive resistance to endothelial stress. In exercised mice, LPS induced far less endothelial apoptosis, and this effect was dependent on the presence of both telomerase...
and eNOS. Exercise also decreased p53 and associated checkpoint kinases and increased the p70 component of the DNA repair enzyme Ku in this study (1943). High shear stress induces increased SIRT1 expression, suggesting exercise may potentially have anti-aging effects in arteries as well. Other effects of exercise on endothelial function including increased PGI₂, thromboxan modulin, and plasmin production also accrue (1145).

The acute increase in blood flow with exercise results in a transient increase in endothelial superoxide anion generation by mitochondria and NADPH oxidase (394). This superoxide is converted rapidly to hydrogen peroxide. This modest, controlled burst of hydrogen peroxide, in turn, also acts to promote increased expression and activity of eNOS, possibly through altered expression of NF-κB as well as stimulation of HSP90 (which strongly supports eNOS activity) through stimulation of HNF1. Importantly, when human cataras was overexpressed in endothelial cells of exercising mice, thereby blocking hydrogen peroxide signaling, the expected increase in eNOS expression in response to exercise was entirely abrogated (983). Endothelial cells adapt to laminar flow by stimulating a number of antioxidant defenses (at least in part through Nrf2 signaling) resulting in increased expression of ecSOD (SOD3) and Cu/Zn SOD (SOD1) as well as a decrease in NADPH oxidase (394). Similar adaptive changes occur with exercise training, explaining the longer term antioxidant effects of exercise despite acute modest stimulation of superoxide and hydrogen peroxide production during acute bouts of exercise (457, 983). Parallel benefits from exercise on insulin signaling pathways in skeletal muscle appear to be blocked by large doses of oral antioxidants, suggesting potential deleterious effects of such interventions (1490). Finally, exercise was shown to clearly reduce atherosclerosis in ApoE-deficient mice together with marked reduction of macrophage and Th1 cell accumulation in the intima. This effect of exercise was entirely blocked by inhibition of eNOS (1335).

D. Hyperlipidemia

Extensive effects of hyperlipidemia to initiate atherosclerosis are reviewed above. Not only does hyperlipidemia activate the endothelium and otherwise promote atherosclerosis, but effects on circulating white blood cells are also apparent. Thus, monocite expression of CCR2, the cognate receptor for MCP-1, was increased twofold when incubated in serum from hypercholesterolemic patients with average LDL of 167 mg/dl compared with persons with LDL of 80 mg/dl (706). This upregulation was thought to be due to uptake of LDL through the LDL receptor and may have been due to an increase in membrane cholesterol. Subsequent studies suggest calcium transients may be involved in this activation with sensitization of monocytes so that they have increased production TNF-α and IL-8 when subse-

quentely exposed to LPS (1293). Native LDL similarly caused calcium transients in endothelial cells with increased VCAM-1 and E-selectin expression (53).

Additional unexpected effects of hypercholesterolemia have been identified. Hypercholesterolemia suppressed the inward rectifying potassium current in endothelial cells (which might be expected to lead to diminished NO response to acute changes in flow) (501). Hypercholesterolemia seems to directly affect PIP₂ sensing amino acids in inwardly rectifying K (Kir2) channels (482). Interestingly, patients with familial hypercholesterolemia were shown to have reduced glycosaminyl volume which could be partially restored by treatment with rosuvastatin (1202). Diet-induced hyperlipidemia in LDLR-null mice led to impaired Treg numbers both in the blood and in atherosclerotic lesions. Treg levels could be restored after 4 wk of chow diet (1112). Oxidized LDL promoted FoxP3 gene methylation by DNA methyltransferases 3a/b, thereby suppressing FoxP3 expression, and may contribute to the decrease in Treg seen in human CAD patients (851).

E. Diabetes

While several other risk factors can recapitulate a form of endothelial activation which, in some settings would be advantageous (such as in infection or physical injury), the endothelial response in diabetes is essentially maladaptive in any setting and finds few if any parallels in normal physiology. Therefore, the term endothelial dysfunction might be considered most apt in diabetes. Unlike other risk factors, hyperglycemia leads to endothelial dysfunction that is systemic, resulting in both aggravation of atherosclerosis and a microvascular disease that is unique to diabetes. Mouse models of type 1 diabetes in hyperlipidemic mice clearly show an excess of atherosclerosis but also increased hyperlipidemia which complicates the interpretation of the isolated effect of increase blood glucose (867, 2102). In type II diabetes, excess free fatty acid exposure and insulin resistance appear to further exacerbate endothelial dysfunction (2000).

Incubation of human endothelial cells with high glucose (770, 815, 2048) or fatty acids (2000) promotes mitochondrial ROS and peroxynitrite (and other RNS) production, leading to ERK1/2 and JNK1 activation, mitochondrial fission and eventual apoptosis. Some of the apparent downstream consequences of this excess ROS and RNS production in the face of high glucose were previously noted and include: uncoupling of eNOS and consumption of NO to produce peroxinitrite; nitration and inhibition of PGIS with diversion of PGH₂ to inflammatory TP signaling and adhesion molecule expression (2123); inhibition of protective ERK5 (1977); increased 12-S-HETE production by 12/15-LO together with proinflammatory fibronectin presentation (1395, 1396); increased expression of BMP4 and
ATP synthesis through F0F1 complexes. The high NADH/H+ ratio would be expected to inhibit protective SIRT1. Regarding OPN regulation, incubation of VSMC with glucose ranging from 5 to 30 mM (90–540 mg/dl) resulted in increased release of UTP and UDP (by an unknown mechanism) with a sigmoidal dose response such that essentially the entire response occurred in the range of 11.5 mM (207 mg/dl) to 20 mM (360 mg/dl) (1309). Extracellular UTP and UDP act on purinergic P2Y2 and P2Y6 receptors to increase calcium entry, thus stimulating calcineurin and nuclear transport of NFATc3 which then stimulates transcription of OPN (1308). Many further insights have emerged from a focus on the excess endothelial generation of ROS and RNS in diabetes.

1. Overabundance fuels the fire of endothelial inflammation and dysfunction in diabetes

An elegant, unified model to explain the seemingly disparate aspects of endothelial dysfunction in diabetes has been forwarded (215, 216, 612). The endothelial cell is one of a handful of cell types that are unable to regulate glucose entry. Glucose moves into the cells through GLUT1 transporters at a rate proportional to external concentrations. Hyperglycemia thus leads to high intracellular glucose, a rapid flux of substrate through the glycolytic pathway, and an abundance of pyruvate for transfer into mitochondria. Synthesis of acetyl CoA ensues followed by ample generation of reducing equivalents (NADH, FADH2) by the citric acid cycle. The transfer of high energy electrons from NADH and FADH2 to complexes of the electron transport chain provides energy to pump hydrogen ions from the matrix into the mitochondrial intermembrane space, thereby generating the hydrogen ion gradient which subsequently drives mitochondrial intermembrane space, thereby generating energy to pump hydrogen ions from the matrix into the complexes of the electron transport chain provides energy to pump hydrogen ions from the matrix into the complexes of the electron transport chain.

Not only does elevated glucose provide a substrate for oxidation and ROS production, but in some way, metabolism of glucose prior to pyruvate entry into mitochondria causes a major shift in mitochondrial morphology from elongated tubelike structures to smaller fragments. Mitochondrial fission and fusion are fundamental features of mitochondrial biology, being intimately involved in distribution of mitochondria into dividing cells, mitochondrial turnover, apoptosis, mitophagy, and ATP generation (583, 1615, 2044). Fission is triggered by phosphorylation events on the cytoplasmic GTPase dynamin-like protein 1 (DLP1) causing binding to its target Fis1 (fission 1) which is anchored on the outer mitochondrial membrane. Inhibition of mitochondrial fragmentation by various measures prevents the increase in ROS produced by cells exposed to excess glucose (1615, 2047, 2048). Glucose-induced mitochondrial fragmentation appears to involve rapid early calcium entry into the cell leading to ERK1/2 activation, followed by phosphorylation of DLP1 and mitochondrial fission. Pyruvate supplies the metabolic fuel to generate ROS by the fragmented mitochondria (2047, 2048). Mitochondrial fission and ROS production occur within 15–30 min after exposure of cells to high glucose, followed by a rapid reduction in mitochondrial fission and ROS production only to be followed by a more prolonged secondary rise in mitochondrial fragmentation and ROS production peaking at ~16 h (2048).

Remarkably, incubation of endothelial cells in high glucose (25–30 mM) for 16 h then returning the cells to normal 5 mM glucose resulted in elevated ROS production that persisted for as long as 6 days. With some cells, only 6 h exposure was sufficient to cause persistent ROS production (203, 467). This elevated ROS production was accompanied by altered histone methylation that favored induction of the p65 component of NF-κB and endothelial activation (203, 467). The mechanism(s) responsible for this “hyperglycemic memory” is under intense investigation and may include the following: mitochondrial dysfunction perpetuated by mitochondrial fission (583, 2044); activation of PP2A by ROS, RNS, and ceramide (especially with increased availability of palmitate) followed by PP2A-mediated dephosphorylation and inhibition of Akt with activation of apoptotic proteins such as Bad (inhibited by Akt), as well as inhibition of AMPK (450, 944); overactive GSK3β blocking assembly of a mitochondrial complex which consists of voltage-dependent anion channel (VDAC), hexokinase (HK), and adenosine nucleotide transporter (ANT) which is responsible for normal shuttling of ADP back into the mitochondria after phosphorylation of glucose (1392, 1495); mitochondrial localization of p66Shc after its phosphorylation by PKCβII (246, 1377, 1712); and impaired SIRT1 activity (2098) with impaired suppression of p66Shc BMP2 and their receptors (185); premature endothelial senescence with inhibition of SIRT1 (210); oxidation of vWF preventing its proteolysis and causing predisposition to thrombosis (1328); and induction of the inflammatory cytokine OPN (1547). Regarding OPN regulation, incubation of VSMC with glucose ranging from 5 to 30 mM (90–540 mg/dl) resulted in increased release of UTP and UDP (by an unknown mechanism) with a sigmoidal dose response such that essentially the entire response occurred in the range of 11.5 mM (207 mg/dl) to 20 mM (360 mg/dl) (1309). Extracellular UTP and UDP act on purinergic P2Y2 and P2Y6 receptors to increase calcium entry, thus stimulating calcineurin and nuclear transport of NFATc3 which then stimulates transcription of OPN (1308). Many further insights have emerged from a focus on the excess endothelial generation of ROS and RNS in diabetes.
by SIRT1 (2106) and decreased FOXO1-mediated induction of MnSOD (1236). Clearly, excess ROS production by mitochondria and probably other sources such as various NOX enzymes is a critical step in triggering endothelial dysfunction in diabetes, but further discussion of its mechanisms is beyond the scope of this review.

2. Consequences of ROS overproduction: genetic damage

While superoxide anion does not penetrate membranes, a substantial amount of the excess superoxide formed on the outer side of the mitochondrial inner membrane can be transferred to the cytosol through the VDAC which traverses the outer mitochondrial membrane (2068). The large increase in endothelial ROS production in diabetes is sufficient to overwhelm SOD antioxidant defenses and results in both nuclear and mitochondrial DNA damage such as single-strand breaks.

Stimulating expression of Nrf2 (by inactivating Keap1 with sulforaphane, a naturally occurring substance in broccoli) upregulated antioxidant defenses and led to a marked reduction in the adverse effects of hyperglycemia, further supporting the role of ROS in mediating endothelial dysfunction in diabetes (2002). α-Lipoic acid is an important mitochondrial antioxidant, and supplementation was shown to decrease atherosclerosis in hyperlipidemic, diabetic mice (2036). Heterozygous deficiency of lipoic acid synthase in diabetic mice resulted in a 48% increase in atherosclerosis (2036). The large increase in endothelial ROS production in diabetes is sufficient to overwhelm SOD antioxidant defenses and results in both nuclear and mitochondrial DNA damage such as single-strand breaks.

In response to DNA damage, the enzyme poly(ADP-ribose) polymerase 1 (PARP-1) is activated. PARP-1 uses NAD⁺ as a substrate to add long ADP-ribose chains to various proteins and transcription factors, to itself, and importantly, to histones. ADP-ribosylation of histones alters their conformation and allows greater access to damaged DNA for repair enzymes. Hydrogen peroxide, although less reactive than other ROS, can also activate PARP-1 (perhaps through formation of highly reactive hydroxyl radical), but to a lesser extent than superoxide and peroxynitrite (1153). PARP-1 is an intriguing enzyme that is rapidly activated 500-fold by binding to single-strand DNA breaks. PARP-1 seems to be a key determinant of three fundamental cellular responses to DNA damage. After mild DNA damage, PARP-1 promotes DNA repair and seems generally protective. With more severe damage, apoptosis is triggered and caspases cleave and inactivate PARP-1, thereby sparing NAD⁺ to function in metabolic pathways to generate ATP to fuel subsequent apoptosis. However, with extensive DNA damage, as apparently occurs with extensive exposure to ROS, PARP-1 is overactivated, with the result that cell stores of NAD⁺ become so extensively depleted that energy generation fails and cellular necrosis ensues. There is evidence that such excessive PARP-1 activation may be involved in myocardial and cerebral reperfusion injury and several PARP-1 inhibitors are in clinical trials for these conditions (1365). Pharmacological inhibition or genetic deletion of PARP-1 leads to a decrease in inflammation and a reduction in atherosclerosis in hyperlipidemic mice (1361, 1889).

3. Compounded consequences of fuel overload in diabetes

Overactivation of PARP-1 appears to be central to the endothelial dysfunction of diabetes (1104, 1659). Surprisingly, among the proteins ADP-ribosylated by PARP-1 in the nucleus is the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH shuttles in and out of the nucleus and appears to function in some way in nuclear DNA repair and NAD⁺ sensing. ADP-ribosylation in diabetic endothelial cells results in GAPDH inhibition in the face of high glycolytic flux, resulting in substrate spill-over into a number of key, deleterious pathways. The substrate for GAPDH is glyceraldehyde-3-phosphate. Elevated glyceraldehyde-3-phosphate leads to accumulation of strongly reactive methylglyoxal. This abnormal byproduct of impaired glycolysis results in rapid synthesis of AGEs by chemical reactions that are far more efficient than glucose-dependent pathways.

Glyceraldehyde-3-phosphate is maintained in equilibrium with dihydroxyacetone phosphate, and in diabetes both are converted at increased rates to glycerol-phosphate, the precursor to DAG. DAG is a direct activator of PKC (particularly the β and δ isofoms but also, the more recently recognized atypical θ isorm which can also be activated by palmitate infusion) (100). Among the numerous proinflammatory actions mediated by activated PKC are enhanced activation of NF-κB, stimulation of NADPH oxidase with increased superoxide anion production and formation of peroxynitrite, depletion of the essential cofactor for eNOS, tetrahydrobiopterin, and resulting impaired NO synthesis and uncoupling of eNOS leading to ROS formation (681).

Impaired movement of intermediates through the glycolytic pathway also leads to elevated fructose-6-phosphate. This leads to increased substrate flux through the hexosamine pathway with production of N-acetylglucosamine (GlcNac) and activated UDP-GlcNac. UDP-GlcNac can form O-GlcNac bonds with serine and threonine, thereby altering the function or activity of a variety of proteins including transcription factors. The result in endothelial cells is decreased activation of eNOS, O-GlcNac modification of IRS-1 and IRS-2 leading to impaired insulin signaling through PI3K and Akt to activate eNOS, increased expression of matrix metalloproteinases (MMPs) together with...
decreased production of tissue inhibitor of metalloproteinase (TIMP), and increased cell mitogenesis (974). Glucosamine supplementation (5% in drinking water) was recently shown to increase ER stress similarly to the presence of diabetes and markedly increased atherosclerosis in ApoE-deficient mice (129). The effect was largely blocked by valproate, an agent that decreases ER stress. Finally, high intracellular glucose stimulates the sorbitol or polyol pathway with depletion of NADPH which is needed to regenerate intracellular glutathione, further exacerbating the pro-oxidant state (216).

If all these actions stemming from PARP-1 deactivating GAPDH were not enough, several studies have pointed to direct, potent proinflammatory and pro-oxidant effects of PPAR-1 itself, possibly by alteration of histones involved in transcription of inflammation-related genes (1323). Cell adhesion molecules VCAM-1, E-selectin, and P-selectin are induced by such PPAR-1 signaling (1889). Other endothelial changes accompanying hyperglycemia include loss of glycocalyx, greater TXNIP-mediated inhibition of Trx (1572), increased LOX-1 expression, increased signaling through RAGE, and direct adverse effects of AGE (through, for example, modification of extracellular matrix proteins).

4. Possible adverse signaling consequences of insulin resistance

Not only does insulin resistance in adipose tissue lead to excessive release of adipose free fatty acids and exposure of the endothelium to excess free fatty acids, but, as noted above, insulin resistance is imposed on endothelial cells, both through ADP-ribosylation of IRS-1 and -2 and through serine phosphorylations of IRS proteins by PKC isoforms and IKK2. This leads to diminished signaling through the PI3K and Akt pathway. But there is another pathway for insulin receptor signaling in endothelial cells. Insulin activates ERK1/2 through the Shc, Grb2-SOS, Ras pathway independent of IRS1/2. While activation of ERK1/2 appears to be protective in some contexts (for example, by phosphorylating eNOS), it can also result in stimulation of NOX5 as well as endothelin-1 release and vasoconstriction. Indeed, when NO production is blocked in wild-type mice, insulin causes vasoconstriction (100).

There is growing evidence for selective insulin resistance resulting in diminished vasodilation through the Akt pathway while the vasoconstricting ERK1/2 pathway is left intact. The vasoconstriction seems to be particularly promoted by the activation of PKCθ by fatty acids, such as palmitate. PKCθ both inhibits insulin signaling through IRS-1 and Akt and promotes signaling through the ERK1/2 pathway (100). However, as depicted in Figure 11, ERK1/2 can also have antioxidant and anti-inflammatory effects. Thus, whether this pathway promotes atherosclerosis in diabetes remains controversial. Additionally, insulin was shown to induce expression of VCAM-1 in endothelial cells by signaling through the insulin-like growth factor 1 receptor while induction of ICAM-1 by insulin was mediated by the insulin receptor. For both, induction could be completely abrogated by inhibiting the MAPK, p38. MEK1 appeared to be involved for both but primarily by activating p38 rather than ERK1/2 (1022).

In an important series of experiments, C57Bl6 mice were fed a high-fat diet (45% calories fat, rich in lard). This caused greater weight gain than chow, a nearly threefold increased level of plasma free fatty acids, increased fasting and stimulated insulin, and hypertension. In endothelium from these animals, insulin was found to stimulate phosphorylation of ERK1/2 as expected, but surprisingly, phospho-Akt increased normally as well. However, there was clearly impaired eNOS phosphorylation and production of NO in response to insulin. In vitro studies further demonstrated impaired eNOS S1177 and S617 phosphorylation at baseline and in response to insulin when cultured endothelial cells were exposed to saturated free fatty acids, particularly palmitate (1723). Again, impaired eNOS phosphorylation was seen despite normal insulin-induced Akt phosphorylation, suggesting either impaired signaling to eNOS or possibly increased phosphorylation activity directed at eNOS. Further studies demonstrated that the impaired eNOS phosphorylation was mediated by ceramide, whose production was stimulated during high-fat feeding or after incubation with palmitate. Ceramide was found to relieve binding of phosphatase PPA2 from an inhibitory cytosolic binding protein and facilitate the direct association of PPA2 with the Akt/eNOS/HSP90 complex. As a result, there was a reduction in the phosphorylation status of eNOS S1177 and S617 and probably Akt as well, together with physical displacement of Akt from the complex (2080). Thus ceramide signaling appears to impair transmission of an insulin signal through Akt to eNOS not by blocking upstream Akt activation but by enhancing phosphatase activity directed at Akt and at least some of its targets.

F. Autoimmune and Inflammatory Disease

Increased risk of atherosclerotic disease has been documented for several autoimmune diseases including systemic lupus erythematosus (SLE) (18, 456, 1500), rheumatoid arthritis or elevated rheumatoid factor (461, 615), systemic sclerosis (904), and psoriasis (601, 635, 1086). It would be reasonable to expect that the elevated plasma cytokines, including TNF-α, IL-6 (1171, 1492), and INF-α/β (994) seen particularly in SLE, would activate endothelial cells (and/or monocytes) and thereby initiate atherosclerotic processes, particularly at atherosclerosis-prone sites. Other mechanisms have recently been forwarded. Multiple causes of Treg dysfunction have been related to both atherosclerosis and autoimmune disease as reviewed above. Similarly, dysfunction of macrophage or DC in clearing apoptotic debris (efferocytosis), or impaired downregulation of in-
Smoking has long been considered an endothelial cell stressor and activator. Cigarette smoke contains 10^{17} free radicals and many other ROS-producing chemicals per puff. Cigarette smoke extract can cause HDAC2 nitration, nitrosylation, and carboxylation, all leading to reduced HDAC2 activity which impairs the normal inhibition of inflammatory gene transcription by HDAC2. Greater NIK activation is also seen (623).

Recently, the effects of exposing cultured endothelial cells to cigarette smoke extract on expression of 35,000 genes was reported (747). There was massive upregulation of genes related to the unfolded protein response, thought to be due to immense oxidative stress. Generation of free radicals was apparently mediated by metals as well as reactive species in the extract. This was accompanied by mitochondrial dysfunction, upregulation of HSF1 and heat shock proteins (including HSP60), and cell cycle arrest. These observations are consistent with prior reports of the pro-oxidant effects of smoking leading to activation of endothelial cells (58). The rapid reversibility of cardiovascular risk after smoking cessation suggests this activation not only affects early endothelial activation but also precipitating factors such as clotting and stability of advanced plaques as well (probably also through inflammatory effects) (946). Nevertheless, some evidence suggests persistent inflammatory effects can last for years after smoking cessation (1927). More recently, the stimulating effects of nicotine on vaso vasorum development seem to contribute to atherosclerosis as well (992) (see below).

**IX. PROMOTION OF ATHEROSCLEROSIS**

The promotion of atherosclerosis centers on the development of foam cells. Insudation of lipoproteins into the subendothelial space as well as lipoprotein retention and modification are major drivers of this stage of atherogenesis. The concentration of LDL found in aortic intimal interstitial fluid is proportional to the plasma concentration (2-fold higher) (1647), consistent with the repeatedly validated log-linear relationship between risk of coronary events and LDL plasma concentrations (with a 30% increase in risk associated with a 30 mg/dl linear rise in plasma LDL) (985). APOB-containing lipoproteins other than LDL are also proatherogenic with the order of atherogenicity, roughly as follows (1873): smaller LDL > larger LDL > \( \beta \)-VLDL > IDL > smaller VLDL > larger VLDL. Coronary disease risks in human hyperlipidemias are consistent with this scheme. Patients with homozygous familial hypercholesterolemia (FH) (due to LDLR mutations) have the highest risk for CAD, followed by LDLR heterozygotes, type III patients, then type IV and possibly type V patients. In all these, low HDL increases risk of atherosclerotic events. This scheme is consistent with the markedly increased atherosclerosis of APOBEC-1-LDLR KO mice that not only have increased LDL levels but, on average, smaller LDL particles (1436).

Importantly, \( \beta \)-VLDL, found in type III hyperlipidemia, cholesterol-fed rabbits and dogs, and ApoE-defective mice, are not simply TGRL remnants. Rather, they represent remnants that have remained in circulation sufficiently long to have altered cholesterol-to-triglyceride ratios and have acquired \( \beta \)-mobility on electrophoresis rather than pre-\( \beta \)-mobility normally seen for TGRL (including normal remnants) in the VLDL density range. Furthermore, it is the cholesteryl ester component of these abnormal TGRL that is atherogenic as demonstrated by the fascinating finding that ACAT2 deficiency almost entirely abrogated atherosclerosis in ApoE null mice (see TABLE 8). In ACAT2 deficiency, APOB-containing particle number (both for APOB-48 and APOB-100) was actually somewhat increased, but the particles had markedly less cholesteryl ester but increased triacylglyceride content (1965). Such lipoproteins were apparently not atherogenic.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Model</th>
<th>%ΔΔA</th>
<th>Function, Comment</th>
</tr>
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<tbody>
<tr>
<td>APOBEC-1</td>
<td>APOBEC-1&lt;sup&gt;−/−&lt;/sup&gt; LDLR&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑504%</td>
<td>APOBEC-1 (APOB editing catalytic polypeptide 1) deaminates a cytosine in APOB mRNA causing change of glutamine 2153 to a stop codon with the resulting APOB being 48% (APOB-48) the size of full length APOB (APOB-100). APOBEC-1 LDLR double KO mice have sufficiently increased LDL that plaques develop on chow diet (results in male mice given) (1436). However, the LDL-size particles that accumulate may also illustrate the greater atherogenicity of smaller LDL as compared to larger TGRL and TGRL remnants.</td>
</tr>
<tr>
<td>ACAT2</td>
<td>ACAT2&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↓98%</td>
<td>ACAT2 (acyl-CoA:cholesterol acyltransferase 2, also known as SOAT2 for sterol O-acyltransferase 2) esterifies cholesterol with fatty acids in intestine and liver whereas ACAT1 functions primarily in macrophages and adrenal cortex. Particle numbers were relatively unchanged in ACAT2 deficient, apoE-null mice but APOB-containing lipoproteins lacked core cholesteryl ester while triglyceride content was increased (1965). Results suggest cholesteryl ester in the triglyceride-rich remnants that accumulate in apoE KO mice is the atherogenic component rather than triglyceride.</td>
</tr>
<tr>
<td>SREBP-1</td>
<td>SREBP-1&lt;sup&gt;−/−&lt;/sup&gt; LDLR&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↓71%</td>
<td>TG levels were 71% lower in SREBP-1 deficient mice on Western diet while total cholesterol was only modestly lower. The cholesteryl content of chylomicrons and VLDL was markedly reduced. In the same study, hepatic over-expression of SREBP-1 increased atherosclerosis 140% while BMT studies had no effect (882).</td>
</tr>
<tr>
<td>APOC1</td>
<td>human APOC1 Tg in apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑42%</td>
<td>Increased TGRL generally likely led to the increase in atherosclerosis in this model (346). Nevertheless, delayed clearance (by an effect of free or HDL-bound apo CI to inhibit hepatic lipase) raises the possibility of more atherogenic composition in humans.</td>
</tr>
<tr>
<td>APOC3</td>
<td>human APOC3 Tg in LDLR&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑165%</td>
<td>Hyperlipidemia was clearly worsened in apo CIII Tg animals (1151). Nevertheless, Apo CIII increased susceptibility to pro-atherogenic modification of LDL by sphingomyelinases with increased adhesion to proteoglycans (768) and has other pro-atherosclerotic effects independent of lipoprotein levels (892).</td>
</tr>
<tr>
<td>CETP</td>
<td>Simian CETP Tg in C57BL/6 fed atherogenic diet</td>
<td>↑517%</td>
<td>CETP (cholesteryl ester transfer protein) expression loaded VLDL and LDL with cholesteryl ester at the expense of HDL in this study (1143). Atherosclerosis extent was strongly and directly proportional to the ratio (LDL-C + VLDL-C)/HDL-C. In the setting of human LCAT over-expression, CETP actually decreased atherosclerosis (137). Thus, relevance to human is unclear.</td>
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**Native lipoprotein affinity for the intimal ECM (extracellular matrix)–lipoprotein and matrix properties**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Model</th>
<th>%ΔΔA</th>
<th>Function, Comment</th>
</tr>
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<tbody>
<tr>
<td>APOB</td>
<td>APOB Tg with proteoglycan binding defect</td>
<td>↓65%</td>
<td>APOB-100 is the structural lipoprotein of VLDL and LDL. 3 forms of proteoglycan binding-defective transgenic APOB with or without defective LDL binding were compared. Proteoglycan binding deficiency resulted in substantial reduction of atherosclerosis at comparable LDL levels (1638).</td>
</tr>
<tr>
<td>LEPR</td>
<td>LEPR&lt;sup&gt;−/−&lt;/sup&gt; rats fed 1% cholesteral</td>
<td>↑290%</td>
<td>Corpulent (cp/cp) rats have a natural mutation causing complete absence of LEPR. The measure of atherosclerosis was unconventional and consisted of the sum of reported myocardial ischemic lesions. Increased binding and retention of intestinal lipoproteins due to increased biglycan content of the arterial wall seemed to be the major differences in this unusual model of severe metabolic syndrome with high insulin levels (1133).</td>
</tr>
<tr>
<td>Perlecan</td>
<td>perlecan&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↓48%</td>
<td>Given is the mean effect in males and females at 12 wk. Later assessments were nonsignificant in both these apoE KO as well as LDLR null mice (1880). Similar reductions seen in apoE KO mice lacking exon 3 of the perlecan gene (1805).</td>
</tr>
<tr>
<td>Decorin</td>
<td>Decorin Tg apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↓47%</td>
<td>Adenovirus mediated transgenic over-expression was seen primarily in liver with increased plasma decorin levels. Shown is the effect from early treatment. Late treatment decreased atherosclerosis as well (37).</td>
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Continued
### Table 8.—Continued

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<tr>
<th>Gene</th>
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<tbody>
<tr>
<td>sPLA2 (group IIa)</td>
<td>PLA2G2A Tg BMT into LDLR −/−</td>
<td>↑ 57%</td>
<td>Macrophages from Tg group IIa sPLA2 overexpressing mice had 2.5-fold greater rates of LDL oxidation, dependent on 12/15 LO expression and increased foam cell formation. Urinary isoprostanes were also increased (1793). Confirmatory of prior observations (833, 1931).</td>
</tr>
<tr>
<td>sPLA2 (group III)</td>
<td>PLA2G3 Tg, apoE−/−</td>
<td>↑ 125%</td>
<td>Secreted phospholipase A2 sPLA2 enzymes modify LDL and promote foam cell formation. Transgenic over-expression of human IIIl sPLA2 caused increased lysophosphatidyl choline in LDL and increased atherosclerosis in animals fed an atherogenic diet (1540).</td>
</tr>
<tr>
<td>sPLA2 (group V)</td>
<td>PLA2G5 −/− BMT into LDLR −/−</td>
<td>↓ 38%</td>
<td>sPLA2 is thought to be largely secreted from activated macrophages. Overexpression of the GV sPLA2 in this same study resulted in a 170% increase in lesion extent as well (186).</td>
</tr>
<tr>
<td>sPLA2 (group X)</td>
<td>PLA2G10 −/− BMT into LDLR −/−</td>
<td>↑ 107%</td>
<td>The main target of this isoform is phosphatidyl choline. Unexpectedly, deficiency of the group X sPLA2 resulted in a marked increase in Th1 activity, IFN-γ, and macrophage necrosis while over-expression of human PLA2G10 reduced plaque size, and enhanced Th2 and macrophage IL-10 production (29).</td>
</tr>
<tr>
<td>SMS2</td>
<td>SMS2−/− apoE−/−</td>
<td>↓ 52%</td>
<td>Sphingomyelin synthase 2 (SMS2) KO mice had lower sphingomyelin content in non-HDL lipoproteins, less aggregation of these lipoproteins upon exposure to sphingomyelinase, and decreased lipoproteins in the aortic wall (437). Similar results with macrophage-only SMS2 deficiency in LDLR −/− mice (1058). Adenoviral delivery of SMS2 to apoE KO mice increased atherosclerosis but also increased non-HDL-C and decreased HDL-C (1914). Similarly, adenoviral expression of SMS1 and SMS2 increased sphingomyelin content and aggregation of APOB-containing lipoproteins after sphingomyelinase treatment (434).</td>
</tr>
<tr>
<td>ASM (secreted)</td>
<td>ASM−/− LDLR−/−</td>
<td>↓ 55%</td>
<td>Acid sphingomyelinase [ASM [causes Niemann-Pick disease, type A when deficient]] also has a secreted form besides the lysosomal form. Secreted ASM cleaves sphingomyelin to ceramide, promotes lipoprotein binding and retention, and greatly increases uptake and foam cell formation for a number of lipoproteins (417).</td>
</tr>
<tr>
<td>LPL</td>
<td>LPL−/− BMT in LDLR−/−</td>
<td>↑ 33%</td>
<td>Macrophage-expressed LPL may promote remnant uptake and retention by bridging lipoproteins and proteoglycans (94). Consistent with similar study in fat fed B6 mice (1850).</td>
</tr>
<tr>
<td>Fhx2</td>
<td>BALB/cJ mice fed a high-fat diet</td>
<td>↓ 97%</td>
<td>Ffh2 (zinc fingers and homeoboxes 2) is a transcription repressor of many genes, including LPL in liver. The BALB/cJ strain of mouse has a defect (a large intron insertion) in Fhx2 that results in very low Fhx2 expression and marked increase in liver LPL expression. As a result, VLDL are cleared more quickly, serum cholesterol is lower, and HDL higher while on a high fat atherogenic diet and atherosclerosis was reduced 30-fold. The degree of atherosclerosis protection appeared greater than could be explained by the lipid changes and there may have been macrophage effects as well (593, 1915).</td>
</tr>
<tr>
<td>ANGPTL3</td>
<td>ANGPTL3−/− apoE−/−</td>
<td>↓ 69%</td>
<td>ANGPTL3 (angiopeptin-like 3) is secreted by liver and inhibits LPL by promoting its cleavage (by proprotein convertases furin and PACE4–paired amino acid converting enzyme 4) (1056). ANGPTL3 also inhibits HL (hepatic lipase) and EL (endothelial lipase). The reduction in atherosclerosis in this model appears almost entirely mediated by the reduction in VLDL and remnants due to a marked increase in LPL activity (62). However, limited human evidence suggests a possible nonlipid mediated effect on carotid plaque (732). Loss of function variants for ANGPTL3, 4, and 5 were associated with lower triglycerides in human populations (1504).</td>
</tr>
<tr>
<td>ANGPTL4</td>
<td>ANGPTL4−/− apoE−/−</td>
<td>↓ 75%</td>
<td>ANGPTL4 inhibits LPL by reversing the dimerization of LPL (2041). Reduction in atherosclerosis presumably was largely due to decreased fasting and postprandial TGRL. However, macrophages from ANGPTL4 KO mice were also shown to accumulate less cholesteryl ester (8).</td>
</tr>
</tbody>
</table>

**Lipoprotein modification, enzymatic (can also affect affinity for ECM)**

- Acid sphingomyelinase (ASM [causes Niemann-Pick disease, type A when deficient]) also has a secreted form besides the lysosomal form. Secreted ASM cleaves sphingomyelin to ceramide, promotes lipoprotein binding and retention, and greatly increases uptake and foam cell formation for a number of lipoproteins (417).

**Macrophage scavenger receptors, bridging proteins, extracellular peptides affecting lipoprotein uptake**

- **LPL**
  - Macrophage-expressed LPL may promote remnant uptake and retention by bridging lipoproteins and proteoglycans (94). Consistent with similar study in fat fed B6 mice (1850).

- **Fhx2**
  - Ffh2 (zinc fingers and homeoboxes 2) is a transcription repressor of many genes, including LPL in liver. The BALB/cJ strain of mouse has a defect (a large intron insertion) in Ffh2 that results in very low Ffh2 expression and marked increase in liver LPL expression. As a result, VLDL are cleared more quickly, serum cholesterol is lower, and HDL higher while on a high fat atherogenic diet and atherosclerosis was reduced 30-fold. The degree of atherosclerosis protection appeared greater than could be explained by the lipid changes and there may have been macrophage effects as well (593, 1915).

- **ANGPTL3**
  - ANGPTL3 (angiopeptin-like 3) is secreted by liver and inhibits LPL by promoting its cleavage (by proprotein convertases furin and PACE4–paired amino acid converting enzyme 4) (1056). ANGPTL3 also inhibits HL (hepatic lipase) and EL (endothelial lipase). The reduction in atherosclerosis in this model appears almost entirely mediated by the reduction in VLDL and remnants due to a marked increase in LPL activity (62). However, limited human evidence suggests a possible nonlipid mediated effect on carotid plaque (732). Loss of function variants for ANGPTL3, 4, and 5 were associated with lower triglycerides in human populations (1504).
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<tr>
<td>LIPG (EL)</td>
<td>LIPG&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↓ 70%</td>
<td>LIPG is EL (endothelial lipase). Mice with LIPG deficiency had higher HDL-C but also higher VLDL-TG (though lower VLDL-C). Macrophage adherence was decreased in vitro in LIPG KO mice (828).</td>
</tr>
<tr>
<td>APOE4 (human E4 isoform versus E3)</td>
<td>human apoE&lt;sup&gt;4/4&lt;/sup&gt; with humanized LDLR BMT into apoE&lt;sup&gt;3/3&lt;/sup&gt; LDLR&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑ 52%</td>
<td>Increase in atherosclerosis is compared with mice receiving apoE&lt;sup&gt;3/3&lt;/sup&gt; with humanized LDLR bone marrow. Thus macrophages with humanized LDLR had twofold increased LDLR expression and apoE&lt;sup&gt;4/4&lt;/sup&gt; lead to increased atherosclerosis as compared with apoE&lt;sup&gt;3/3&lt;/sup&gt; (57).</td>
</tr>
<tr>
<td>HSP27</td>
<td>HSP27 Tg apoE&lt;sup&gt;−/−&lt;/sup&gt; females</td>
<td>↓ 35%</td>
<td>HSP27 (heat shock protein 27) is secreted by macrophages but only upon estrogen receptor stimulation. Also strongly expressed in VSMC. HSP27 binds to SR-A and inhibits uptake of AcLDL. Also, possibly down-regulates SR-A (1452). Tg overexpression resulted in increased plasma HSP27 only in females and dependent upon the presence of estradiol or estrogen receptor agonist (1464). Atherosclerosis was inversely proportional to serum HSP27 (1463).</td>
</tr>
<tr>
<td>MSR1 (SR-AI and SR-AII)</td>
<td>SR-AI/II&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↓ 58%</td>
<td>MSR1 (macrophage scavenger receptor 1) gene gives rise to SR-AI and SR-AII by alternate splicing (1718).</td>
</tr>
<tr>
<td>CD36</td>
<td>CD36&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↓ 45%</td>
<td>CD36 is a class B scavenger receptor (507). As for MSR above, results for KO with this receptor have been somewhat variable (654) but generally confirmatory (716). Confirmed also in LDLR KO BMT (1121). Also decreases thrombogenic potential since platelet CD36 interacts with oxidized phospholipids in hyperlipidemia to promote platelet activation (1426). CD36 signaling through Fyn leads to a CD36:TLR4:TLR6 complex with downstream signaling through both MyD88 and TRIF (1684).</td>
</tr>
<tr>
<td>DGAT1</td>
<td>DGAT1&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↓ 24%</td>
<td>DGAT1 (acyl-CoA:diacylglycerol acyltransferase 1) deficiency in other mice leads to resistance to dietary fat-induced obesity and inflammation. Much of the effect on atherosclerosis may have been due to reduced serum cholesterol and triglycerides, but SR-A expression and foam cell formation was reduced in isolated macrophages from DGAT1 KO mice (273).</td>
</tr>
<tr>
<td>VLDLR</td>
<td>VLDLR&lt;sup&gt;−/−&lt;/sup&gt; LDLR&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>NS</td>
<td>Increased necrosis in plaques in VLDLR KO despite no effect on size. Endothelial over-expression had no effect (1734).</td>
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<tr>
<td></td>
<td>VLDLR&lt;sup&gt;−/−&lt;/sup&gt; BMT into VLDLR&lt;sup&gt;−/−&lt;/sup&gt; C57Bl/6 fed Paigen diet</td>
<td>↑ 170%</td>
<td>Only a nonsignificant 29% decrease in lesion size was seen comparing VLDLR&lt;sup&gt;−/−&lt;/sup&gt; to VLDLR&lt;sup&gt;−/+&lt;/sup&gt; mice (459).</td>
</tr>
<tr>
<td>LDLR</td>
<td>LDLR&lt;sup&gt;−/−&lt;/sup&gt; BMT into C57Bl/6 fed Paigen diet</td>
<td>↓ 66%</td>
<td>Many fewer macrophage foam cells were seen in lesions despite no difference in plasma lipids. Cultured macrophages took up less β-VLDL (754).</td>
</tr>
<tr>
<td></td>
<td>LDLR&lt;sup&gt;−/+&lt;/sup&gt; BMT into LDLR&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>NS</td>
<td>Confirmed by 3 different teams (169, 503, 753).</td>
</tr>
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Extracellular hormone peptides that promote foam cell formation

| GLP-1                 | GLP-1 infusion for 4 wk in apoE<sup>−/−</sup> | ↓ 27%| GLP-1 (glucagon-like peptide 1) increases cAMP through its receptor on macrophages and VSMC. Effects of infusion of GLP-1 for 4 wk into 17-wk-old apo E null mice on aortic root are shown. GLP-1 treatment inhibited macrophage infiltration into artery walls and reduced expression of CD36. Endothelial expression of MCP-1, VCAM-1, ICAM-1, and PAI-1 were also all suppressed by GLP-1 or GIP (1271). |
| GIP                   | GIP infusion in apoE<sup>−/−</sup> | ↓ 39%| GIP (gastric inhibitory peptide, also glucose-dependent insulinotropic polypeptide) had effects comparable to GLP-1 (1271). |
| Salusin-α             | Salusin-α infusion in apoE<sup>−/−</sup> | ↓ 50%| Salusins are peptides alternatively processed from the T0R2A (torsin 2A) gene. They are synthesized in a variety of tissues including the vasculature and are found in plasma. Salusin-α inhibits foam cell formation in vitro and with infusion. Shown are results of 8 wk of infusion (1270). |
| Salusin-β             | Salusin-β infusion in apoE<sup>−/−</sup> | ↑ 90%| Salusin-β promotes foam cell formation and VSMC proliferation but also has pro-proliferative and anti-apoptotic effects on cardiomyocytes. Shown are effects on aortic root atherosclerosis of 4 wk infusion starting at 13 wk. Macrophage CD36 expression was upregulated (1270). |

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<tr>
<td>LXR [α and β]</td>
<td>LXRαβ−/− BMT to LDLR−/−</td>
<td>↑230%</td>
<td>LXR has multiple effects both on both macrophage scavenger receptor expression and lipoprotein uptake and efflux. LXR is also strongly anti-inflammatory (1756). Others report an 83% reduction of atherosclerosis in an LXR transgenic mouse (243, 1776).</td>
</tr>
<tr>
<td>LXRα (NR1H3)</td>
<td>LXRα−/− LDLR−/−</td>
<td>↑73%</td>
<td>Only LXRα (also known as NR1H3 for nuclear receptor subfamily 1, group H, member 3) KO increased atherosclerosis. LXRβ represses ABCA1, ABCG1, and SREBP1c expression in the absence of LXR agonists. Not all the increase in atherosclerosis was due to macrophage deficiency in BMT studies (152).</td>
</tr>
<tr>
<td>FXR</td>
<td>FXR−/− apoE−/− on high fat diet</td>
<td>↓54%</td>
<td>FXR (farnesoid X receptor) is also known as NR1H4 (nuclear receptor subfamily 1, group H, member 4). Female apoE KO mice have more atherosclerosis and showed reduction with FXR KO (674). Macrophages expressed less CD36 with FXR KO but more TNF-α and INF-γ. In LDLR KO model, males have more atherosclerosis and showed reduction with FXR KO (2085).</td>
</tr>
<tr>
<td>LXRβ (NR1H2)</td>
<td>LXRβ−/− LDLR−/−</td>
<td>NS</td>
<td></td>
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<tr>
<td>iVP16LXRα LDLR−/−</td>
<td>↓41%</td>
<td>Intestine-specific expression of a constitutively active LXRα (iVP16LXRα) increased nonbile trans-intestinal cholesterol excretion (TICE) and enhanced reverse cholesterol transport from macrophages to intestine while decreasing liver fat content (1066).</td>
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</tr>
<tr>
<td>Arg1</td>
<td>Arg1 variant with increased activity in cholesterol-fed rabbits</td>
<td>↓41%</td>
<td>Arg1 (arginase 1) variant in atherosclerosis resistant rabbits was shown to have greater activity (1775, 1785). Arg1 is greatly increased in regressing plaques and produces polyamines which promote resolution of inflammation and reduces INOS production of NO in macrophages. Arg1 is a downstream target of LXRα. LXRα first induces IGF8 which then acts as a transcription factor for Arg1 (1435).</td>
</tr>
<tr>
<td>FXR</td>
<td>FXR−/− apoE−/− on high fat diet</td>
<td>↑104%</td>
<td>In another study FXR−/− apoE−/− mice displayed severe steatohepatitis, reduced weight gain, and lower survival. VLDL and LDL were increased and HDL reduced (710). In both studies, only high-fat, high-cholesterol fed mice showed differences in atherosclerosis. FXR effects on atherosclerosis are complex but appear primarily dependent on resulting serum lipid levels (727). FXR KO may have unexpected effects compared to FXR agonists (690).</td>
</tr>
<tr>
<td>PPARα</td>
<td>PPARα−/− BMT into LDLR−/−</td>
<td>↑25%</td>
<td>Given is approximate mean of male (44% increase) and female (3-5% increase, NS) for proximal aorta (92). Greater effects for distal aorta (both genders about 45% increase). Blood pressure (decreased) and metabolic effects (higher triglycerides, more insulin sensitive) made results difficult to interpret for whole body PPARα−/− apoE−/− model (1802).</td>
</tr>
<tr>
<td>PPARδ</td>
<td>PPARδ−/− BMT into LDLR−/−</td>
<td>↓62%</td>
<td>The decreased atherosclerosis was surprising given a substantial decrease in atherosclerosis also seen with a PPARδ agonist (988). Additional studies led to a model in which unliganded PPARδ binds Bcl6 while PPARδ ligand displaces Bcl6, freeing it to act as a corepressor for many proinflammatory genes. Thus either PPARδ agonist or complete absence of PPARδ results in more free Bcl6 and greater co-repression of pro-inflammatory genes. This model has been validated in subsequent studies (103, 286, 1743). Bcl6 acts in opposition to NF-κB for over 1000 genes (109).</td>
</tr>
<tr>
<td>Bcl6</td>
<td>Bcl6−/− BMT into LDLR−/−</td>
<td>↑57%</td>
<td>Bcl6 (B-cell lymphoma 6) is a DNA binding protein which inhibits transcription when interacting with SMRT, NCoR and other corepressor complexes. These downregulate transcription of some 20-30,000 genes (with 50% overlap), many being pro-inflammatory NF-κB target genes (110). It is bound by unoccupied PPARδ.</td>
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<td>PPARγ</td>
<td>PPARγ&lt;sup&gt;−/−&lt;/sup&gt; BMT into apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑ 30%</td>
<td>The PPARγ induces LXR and CD36 transcription while LXR induces ABCA1 (287). Similar results in LDLR&lt;sup&gt;−/−&lt;/sup&gt; mice (97). PPARγ also promotes macrophage efferocytosis (286). PPARγ signaling also normally suppresses atherosclerosis-associated vascular calcification by a newly recognized Wnt5a pathway in VSMC (1969).</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>PGC-1α&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>NS</td>
<td>No change in lesions despite more macrophages and increased ICAM-1 expression in plaques (1879). The PGC-1α&lt;sup&gt;−/−&lt;/sup&gt; mice were lean and had lower serum triglycerides but had much lower aortic PPARα and PPARγ expression.</td>
</tr>
<tr>
<td>AEBP1</td>
<td>AEBP1 Tg BMT into apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑ 280%</td>
<td>AEBP1 (adipocyte enhancer-binding protein 1) is a transcriptional repressor that downregulates LXR and PPARγ activity in macrophages (1116). Parallel studies including deletion of AEBP1 and studies in LDLR KO animals were confirmatory (163).</td>
</tr>
<tr>
<td>Nrf2 (NFE2L2)</td>
<td>Nrf2&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↓ 27%</td>
<td>Nrf2, also known as NFE2L2 (nuclear factor erythroid type 2-like 2), supports expression of CD36 and foam cell formation in macrophages (1717). Chow-fed Nrf2 KO mice again showed less CD36 in macrophages, fewer macrophages in plaques, but also lower plasma lipid levels and reduced hepatic lipid production (106).</td>
</tr>
<tr>
<td>ABCB4</td>
<td>ABCB4&lt;sup&gt;−/−&lt;/sup&gt; BMT into LDLR&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑ 81%</td>
<td>ABCB4 (also known as P glycoprotein 3) Foam cell formation was greatly accelerated upon incubation with acetylated LDL due to increased activity of scavenger receptors. Neither cholesterol nor phospholipid efflux to HDL were affected (1403).</td>
</tr>
<tr>
<td>Rip2</td>
<td>Rip2&lt;sup&gt;−/−&lt;/sup&gt; BMT into APOB&lt;sub&gt;100&lt;/sub&gt; x LDLR&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑ 37%</td>
<td>Appeared to promote macrophage spreading and macropinocytosis (1012).</td>
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**Macrophage and VSMC intracellular lipid trafficking**

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<tr>
<td>LIPA</td>
<td>injection of human LIPA into LDLR&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↓ 50%</td>
<td>LIPA (lipase A, lysosomal acid, also LAL) cleaves both CE and TG in lysosomes. Another model with LIPA deficiency in apo E KO mice may have had increased atherosclerosis but comparison with apo E KO alone not given (449).</td>
</tr>
<tr>
<td>NPC1</td>
<td>NPC1&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑ 230%</td>
<td>NPC1 (Niemann-Pick disease type C1) is involved in intracellular FC trafficking. NPC1&lt;sup&gt;−/−&lt;/sup&gt; mice also displayed greatly increased thrombosis (1940). However, heterozygous deficiency of NPC1 decreased atherosclerosis with less apoptosis associated with free cholesterol accumulation in the ER (514).</td>
</tr>
<tr>
<td>ACAT1</td>
<td>ACAT1&lt;sup&gt;−/−&lt;/sup&gt; BMT LDLR&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑ 119%</td>
<td>Macrophage-specific KO of ACAT1 impaired cholesteryl esterification in macrophages and decreased macrophage total cholesterol content but greatly increased toxic free cholesterol and total plaque area (504). Also known as SOAT1 (sterol O-acyltransferase 1).</td>
</tr>
<tr>
<td>Elovl6</td>
<td>Elovl6&lt;sup&gt;−/−&lt;/sup&gt; BMT LDLR&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↓ 36%</td>
<td>Elovl6 is a fatty acid elongase and appears to provide preferred substrate (mainly oleate) for ACAT1 to form cholesteryl esters in foam cells (1528).</td>
</tr>
<tr>
<td>ADRP (PLIN2)</td>
<td>ADRP&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↓ 25%</td>
<td>ADRP (adipose differentiation-related protein, also known as perilipin 2) is a major lipid droplet protein required for foam cell formation. Deficiency in macrophages decreased the number of lipid droplets and increased cholesteryl efflux in macrophages (1397).</td>
</tr>
<tr>
<td>ATGL</td>
<td>ATGL&lt;sup&gt;−/−&lt;/sup&gt; BMT into LDLR&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↓ 43%</td>
<td>Macrophages lacking ATGL (adipose triglyceride lipase) had massive TG accumulation but showed less inflammation (978). Mechanism unknown.</td>
</tr>
<tr>
<td>NCEH1</td>
<td>NCEH1&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑ 70%</td>
<td>NCEH1 (neutral cholesteryl ester hydrolase 1) releases free cholesterol (FC) from cytoplasmic lipid droplets and promotes FC efflux. NCEH1-deficient macrophages accumulated more CE when incubated with acetylated LDL, β-VLDL, or oxLDL. Lesions also had more FC and CE (1584).</td>
</tr>
<tr>
<td>CEH</td>
<td>macrophage-specific NCEH1 transgenic LDLR&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↓ 50%</td>
<td>CEH (cholesteryl ester hydrolase, also known as CES1 for carboxylesterase 1) is another enzyme with neutral CEH activity but is distinct from NCEH1. (2086). CEH accounts for 10% or less of neutral CEH activity. Nevertheless, overexpression decreased atherosclerosis and suppressed macrophage NF-κB and AP-1 signaling with less secretion of IL-1, IL-6, and MCP-1 (144).</td>
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<tr>
<td>HSL</td>
<td>HSL&lt;sup&gt;−/−&lt;/sup&gt; BMT into LDLR&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑ 20%</td>
<td>HSL (hormone-sensitive lipase, also abbreviated LIPE for lipase E) also protected from foam cell formation but to somewhat less extent than NCEH1. The 20% increase in atherosclerosis with HSL-null bone marrow was not significant versus wild type. However, HSL&lt;sup&gt;−/−&lt;/sup&gt; NCEH1&lt;sup&gt;−/−&lt;/sup&gt; BMT (100% increase) versus NCEH1&lt;sup&gt;−/−&lt;/sup&gt; BMT only (70% increase) was significant (1584). Thus, the effects of these two lipases were roughly additive.</td>
</tr>
<tr>
<td>GPR109A</td>
<td>GPR109A&lt;sup&gt;−/−&lt;/sup&gt; LDLR&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑ 33%</td>
<td>This is the niacin receptor, a GPCR expressed in macrophages, other white cells, and adipocytes but not endothelial cells. Increased atherosclerosis shown here is a comparison between GPR109A&lt;sup&gt;−/−&lt;/sup&gt; LDLR&lt;sup&gt;−/−&lt;/sup&gt; versus LDLR&lt;sup&gt;−/−&lt;/sup&gt; mice with both on niacin. BMT with GPR109A&lt;sup&gt;−/−&lt;/sup&gt; cells abrogated the anti-atherosclerotic effect of niacin (1087).</td>
</tr>
<tr>
<td>APOA1</td>
<td>APOA1&lt;sup&gt;−/−&lt;/sup&gt; LDLR&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑ 110%</td>
<td>LDLR/APOBEC1-deficient mice express only APOB-100 in LDL. In these APOA1-deficient mice, “HDL-C” was normal but the HDL lacked APOA1 and instead had high apoE. The mice showed impaired ABCA1- and SR-B1-mediated cholesterol efflux and decreased reverse cholesterol transport (1232).</td>
</tr>
<tr>
<td>RORα</td>
<td>RORα&lt;sup&gt;−/−&lt;/sup&gt; in C57BL/6 fed atherogenic diet</td>
<td>↑ 504%</td>
<td>RORα (retinoic acid receptor-related orphan receptor α). This mouse had a profound reduction in HDL, but of interest, this was due to reduction of apolipoprotein production in intestine, not liver (1132).</td>
</tr>
<tr>
<td>APOA2</td>
<td>human APOA1/AlI Tg in C57BL/6 fed atherogenic diet</td>
<td>↑ 1367%</td>
<td>Comparison was with the human APOA1 Tg. The nontransgenic C57BL/6 had about 3 times more atherosclerosis than the APOA1/AlI transgenic (1570). Less striking results in another study (1928). The relevance of these studies to human atherosclerosis remains unknown but human APOA1I seemed to displace PON and rendered mouse HDL proinflammatory and pro-oxidant (264).</td>
</tr>
<tr>
<td>APOA1I</td>
<td>human APOA1I Tg in rabbits fed high cholesterol diet</td>
<td>↓ 56%</td>
<td>The mean reduction in en face aortic lesions in males and females is given. Coronary atherosclerosis was also seen and reduced. HDL of the APOA1I Tg rabbits showed greater cholesterol efflux capacity and ability to reduce inflammatory cytokine production by LPS-treated macrophages and CRP levels were markedly reduced. LDL, VLDL were less oxidizable. There was an increase in preβ HDL compared to larger HDL (1920).</td>
</tr>
<tr>
<td>ABCA1</td>
<td>ABCA1&lt;sup&gt;−/−&lt;/sup&gt; BMT apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑ 58%</td>
<td>Whole body KO of ABCA1 did not affect atherosclerosis but cholesterol levels were reduced. In contrast, bone marrow knockout did increase atherosclerosis as shown here (22). More recently, whole body overexpression decreased atherosclerosis, liver-specific KO increased atherosclerosis (together a large reduction in HDL), while macrophage-specific KO had no effect (220). Infusion of apo E null mice with ABCA1 antibody increased atherosclerosis by 72% (1246).</td>
</tr>
<tr>
<td>TRPV1</td>
<td>TRPV1&lt;sup&gt;−/−&lt;/sup&gt; ApoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑ 300%</td>
<td>TRPV1 (transient receptor potential vanilloid 1) is a calcium channel that mediates burning pain in response to capsaicin stimulation. In TRPV1-null mice there was a reduction of phosphorylated eNOS and NO production, presumably through reduced endothelial calcium entry and reduced CaMKII, PYK2, and ERK1/2 activation (309). This signaling would be expected to be proatherogenic, however. In another study, intracellular calcium elevation mediated by capsaicin in VSMC resulted in downregulation of LR1P and upregulation of ABCA1 with decreased lipid accumulation. Apo E null mice treated with capsaicin and high fat diet had 47% less atherosclerosis while no effect was seen in TRPV1 deficient double KO mice (1100).</td>
</tr>
<tr>
<td>ABCA5</td>
<td>ABCA5&lt;sup&gt;−/−&lt;/sup&gt; BMT into LDLR&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑ 50%</td>
<td>Given is increase lesion size at 6 wk in females. By 10 wk a NS 30% increase was seen. No increase in males was seen. There was impaired transfer of cholesterol to spherical HDL from ABCA5&lt;sup&gt;−/−&lt;/sup&gt; macrophages with compensatory increase in ABCA1 but not ABCG1 (2031).</td>
</tr>
<tr>
<td>ABCA7</td>
<td>ABCA7&lt;sup&gt;−/−&lt;/sup&gt; BMT into LDLR&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>NS</td>
<td>ABCA7 may promote cholesterol efflux and effectorcytosis but no effect of KO was seen on atherosclerosis (937). ABCA7 is upregulated by cholesterol loading. However, ABCA7 deficiency leads to marked compensatory upregulation of both ABCA1 and especially ABCG1 (1201). If anything, ABCA1/ABCA7 double KO BMT had somewhat less atherosclerosis than ABCA1 KO, presumably due to marked ABCG1 upregulation.</td>
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### Table 8.—Continued

<table>
<thead>
<tr>
<th>Gene</th>
<th>Model</th>
<th>%Δ A</th>
<th>Function, Comment</th>
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<tbody>
<tr>
<td>ABCG1</td>
<td>ABCG1&lt;sup&gt;−/−&lt;/sup&gt; ABCA1&lt;sup&gt;−/−&lt;/sup&gt; BMT into LDLR&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑ 141%</td>
<td>Shown is the comparison between mean proximal aortic lesion area in ABCG1&lt;sup&gt;−/−&lt;/sup&gt; + ABCA1&lt;sup&gt;−/−&lt;/sup&gt; BMT to ABCA1&lt;sup&gt;−/−&lt;/sup&gt; BMT, both in LDLR&lt;sup&gt;−/−&lt;/sup&gt; animals. BMT with ABCA1&lt;sup&gt;−/−&lt;/sup&gt; marrow showed modest increase in atherosclerosis but no increase was seen for ABCG1&lt;sup&gt;−/−&lt;/sup&gt; only, consistent with prior studies (2054).</td>
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<tr>
<td></td>
<td>ABCG1&lt;sup&gt;−/−&lt;/sup&gt; LDLR&lt;sup&gt;−/−&lt;/sup&gt; with BMT of wild type marrow</td>
<td>↑ 20%</td>
<td>Plaque increase given is for aortic root; 2.2-fold increase was seen in the arch, both only after 23 wk (1946). Increased EC free cholesterol increased Cav1 interaction with eNOS and impaired NO synthesis which was reversed by HDL (1770).</td>
</tr>
<tr>
<td>ORP1L</td>
<td>ORP1L Tg BMT into LDLR&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑ 108%</td>
<td>ORP1L (oxysterol binding protein (OSBP)-related protein 1L) binds 22- and 25-hydroxycholesterol. ORP1L over-expressing macrophages displayed reduced ABCG1, ABCG5, and APOE expression with decreased cholesterol efflux to spherical HDL₂. PLTP secretion was also increased (2015).</td>
</tr>
<tr>
<td>SR-B1 (SCARB1)</td>
<td>SR-B1&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑ 5000%</td>
<td>SR-B1 is an HDL receptor and can be involved in macrophage cholesterol efflux as reverse cholesterol transport from HDL to liver. Controls had essentially no lesions at 4-7 wk. SR-B1 deficiency also resulted in MI in mice (1808) possibly by activation of platelets due to increased free cholesterol (938).</td>
</tr>
<tr>
<td></td>
<td>SR-B1&lt;sup&gt;−/−&lt;/sup&gt; BMT apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑ 86%</td>
<td>Macrophage-specific deficiency of SR-B1 contributed to the strong proatherogenic effect noted for whole-body knockout. However, cholesterol efflux to HDL from incubated macrophages lacking SR-B1 was not different than wild-type macrophages (2081). SR-B1 plays a major role in the reverse cholesterol transport and decreases atherosclerosis.</td>
</tr>
<tr>
<td>PDZK1</td>
<td>PDZK1&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑ 26%</td>
<td>PDZK1 (PDZ domain-containing protein 1) is not a kinase but a scaffold that helps organize various receptors and ion channels. PDZK1 KO mice have a marked reduction in SR-B1 in liver but not in macrophages or endothelial cells. A fourfold increase (to about 40% of mice) in occlusive lesions (50-100% stenosis) was seen (2034) besides the increased area of atherosclerosis given in this table.</td>
</tr>
<tr>
<td>GNMT</td>
<td>GNMT&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑ 500%</td>
<td>GNMT (glycine N-methyltransferase) synthesizes sarcosine from glycine with a methyl group donated from Sadenosyl methionine. GNMT KO mice had higher cholesterol levels but isolated macrophages showed increased cholesterol accumulation with downregulation of ABCA1, ABCG1, and SR-B1, thus the mechanism for increased atherosclerosis is unclear (291).</td>
</tr>
<tr>
<td>APOM</td>
<td>APOM adenoviral transduction in LDLR&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↓ 78%</td>
<td>Adenovirus-apoM injection led to 3- to 4-fold increase in plasma apo M. Lesions assessed 3 wk later. Apo M increased HDL, decreased HDL catabolism, and promoted increased cholesterol efflux by enhanced formation of pre-β HDL (1970). Similar but less dramatic effects confirmed in a later study using transgenic over-expression of human APOM (321).</td>
</tr>
<tr>
<td>HL (LIPC)</td>
<td>HL&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↓ 73%</td>
<td>HL (hepatic lipase), also known as LIPC (lipase C). Primarily female mice showed decreased atherosclerosis (1204).</td>
</tr>
<tr>
<td>LCAT</td>
<td>LCAT&lt;sup&gt;−/−&lt;/sup&gt; LDLR&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↓ 35%</td>
<td>LCAT (lecithin cholesterol acyltransferase) deficiency decreased atherosclerosis despite a profound reduction in HDL-C (977). Reduced LDL and VLDL-C were also decreased modestly. Reverse cholesterol transport is increased by LCAT deficiency while over-expression of LCAT decreases availability of pre-β HDL, decreases reverse cholesterol transport and increases atherosclerosis (1447).</td>
</tr>
<tr>
<td></td>
<td>LCAT&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↓ 51%</td>
<td></td>
</tr>
<tr>
<td>PLTP</td>
<td>PLTP&lt;sup&gt;+/+&lt;/sup&gt; BMT into PLTP&lt;sup&gt;−/−&lt;/sup&gt; LDLR&lt;sup&gt;−/−&lt;/sup&gt; mice</td>
<td>↓ 48%</td>
<td>PLTP (phospholipid transfer protein) is induced by LXR and secreted locally by macrophages where it interacts with ABCA1 and promotes cholesterol transfer to APOA1 or pre-β HDL. PLTP may also facilitate local regeneration of pre-β HDL. Shown here is effect of restoration of normal macrophage PLTP production in otherwise PLTP-deficient mice (1839). Higher systemic PLTP activity seems to promote APOA1 loss, reduced plasma HDL, and increased atherosclerosis (1856).</td>
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Reference numbers are given in parentheses. Not included are gene modifications which lead to changes in lipoprotein plasma levels (see text).
Interestingly, a subset of human FH patients who have both heterozygous LDLR mutations and an ApoE2 variant (and who clearly manifest type III hyperlipidemia in addition to high LDL) do not have higher risk than other persons with FH without type III hyperlipidemia (260, 783, 786). This may be due to reduced conversion of remnants to LDL and somewhat lower LDL levels in this combined lipid disorder. These FH patients differ from combined LDLR/ApoE-deficient mice which have much more severe atherosclerosis than LDLR KO mice, possibly due to impairment of macrophage effectorcytosis imposed by ApoE deficiency and other lipoprotein abnormalities (for example in HDL) seen in ApoE deficiency (see below). Low levels of HDL are clearly associated with elevated atherosclerosis risk. The common combination of low HDL with elevated triglycerides has been associated with remarkably elevated risk in some studies (782, 785). This repeatedly observed epidemiologic association between low HDL and increased risk of CAD does not obviate the importance of HDL function in addition to HDL concentration. Common variants in at least 95 genes have now been shown in human GWAS studies to affect serum lipid with many also contributing to CAD risk (1774), but these will not be reviewed here.

The notion of postprandial atherogenesis (2117, 2118) remains a strong possibility, particularly in light of the recent report that much more APOB-48 was found in human plaque than APOB-100 (1867). Furthermore, transgenic overexpression of SREBP-1c in LDLR-null mice increased VLDL production rate, VLDL size, and postprandial plasma triglyceride levels, with greater overall exposure to TGRL remnants. These animals suffered increased atherosclerosis. Conversely, double KO of SREBP-1 and LDLR resulted in lower VLDL and less atherosclerosis compared with LDLR KO controls (882). Other genes that may affect lipoprotein size and composition due to effects on synthesis or residence time are apo CI, apo CIII, and CETP with proatherogenic effects demonstrated for all these except CETP, which showed mixed effects (see Table 8).

An intriguing additional theoretical mechanism related to fat intake and chylomicrons is the possibility that these intestinal lipoproteins carry sufficient LPS from intestinal bacteria to promote inflammation in both adipose and the artery wall (237, 1919). However, ApoE-deficient animals had more atherosclerosis when raised in germ free conditions, at least when fed a low cholesterol diet, though there was essentially no difference when the mice were fed a high-fat diet (1683).

Somewhat related is the important recent observation that the intestinal microflora decomposes dietary choline to the gas trimethylamine which is efficiently absorbed and metabolized in the liver by FMO3 (flavin monooxygenase 3 and possibly other related enzymes) to a proinflammatory agent, TMAO trimethylamine N-oxide (TMAO) which promotes inflammation and macrophage foam cell formation. ApoE KO mice fed a high-choline diet had three times the atherosclerosis compared with either antibiotic-treated mice or mice fed a regular diet. Atherosclerosis susceptibility also seemed to correlate with FMO3 activity in mice with different genetic backgrounds. Data demonstrating a strong positive association between CAD and TMAO in human subjects was also presented (1924).

### A. Dendritic Cells as the First Foam Cells

Dendritic cells (DCs) are the earliest inhabitants of the subendothelial intimal space, concentrated clearly in atherosclerosis-prone areas of disturbed flow, as first shown surprisingly in healthy young humans and rabbits (1212). Their presence was already established in all specimens examined even in infancy or early childhood, in the absence of any risk factors, well before the presence of any evidence of macrophage accumulation or lipid-filled cells, and was universally extant in normolipidemic animals as well (161, 1212). Apparently, the degree of endothelial activation with VCAM-1 expression at hemodynamically predisposed sites was sufficient to signal DC to set up their surveillance in these areas as “professional” antigen-presenting cells (864). The DC in these networks were shown capable of antigen presentation, and as other DC, were capable of moving to nearby lymphatic tissue where phagocytosed antigens could be presented to T-cells (1851). Furthermore, hypercholesterolemia does not diminish the ability of DC to prime naive T-cells (1366).

All DC express CD11c (integrin αX, encoded by ITGAX) and MHCII (thus, DC are CD11c±, MHCII±). Temporary ablation of subintimal DCs was achieved in LDLR-deficient mice by selective expression of a diptheria toxin receptor only in CD11c+ cells followed by diptheria toxin administration. When these mice were then fed a high-fat diet, there was a marked reduction in intimal foam cell formation compared with untreated mice, with the difference detectable in just 5–10 days. Thus subintimal DCs seem to represent the first foam cells to be formed in the face of hyperlipidemia (1399). Consistent with this observation, ITGAX deficiency in apoE−/− mice resulted in a modest 24% decrease in atherosclerosis (1986) (see Table 6). KO of CD74, a chaperone required for normal expression of MHCII, decreased atherosclerosis over 70%, but such a defect would have disrupted many aspects of adaptive immunity (see Table 7). The relevance of these findings to the role of DCs in atherosclerosis must be considered in light of additional complexities.

1. **DCs as complex modulators of atherogenesis**

The role of DCs in atherogenesis can be anticipated to be complex, since DCs can orchestrate either the sensitization or anergy of Th cells toward presented antigen as well as the
control of Treg, all depending on the array of displayed costimulatory or coinhibitory receptors and the mix of secreted cytokines. DCs are broadly subdivided into conventional (derived from non-monocyte DC precursors) and nonconventional DCs (consisting of plasmacytoid and monocyte-derived DC).

On the basis of recent work, prelesional, intimal DCs include similar numbers of conventional and monocyte-derived DCs. Plasmacytoid DCs seem to enter into the intima later as lesions progress (314). In fact, all studied DC subsets seem to proliferate or enter the plaque in increased numbers as lesions advance. Conventional DCs proliferate in response to the cytokine FLT3L which binds the DC receptor FLT3 (FMS-related tyrosine kinase 3 where FMS refers to feline McDonough sarcoma). Conventional DCs do not respond to M-CSF. In contrast, monocyte-derived DCs proliferate in response to M-CSF but not FLT3L. Thus administration of FLT3L in test animals only promoted proliferation of prelesional conventional DCs, and these could be tracked by cell-specific markers. Importantly, KO of FLT3 increased atherosclerosis along with a reduction of Treg (314).

A subset of conventional DCs was found to accumulate later in lesions and secrete CCL17 which specifically restrained proliferation and promoted apoptosis of Treg while directing atherogenic CD4+ T-cell responses, all in an antigen-specific manner (1932). Genetic deletion of CCL17 reduced atherosclerosis in ApoE-deficient mice (1932) (see Table 7).

Possibly, the conversion of DCs to early foam cells and the reduction of atherosclerosis from general DC depletion noted above represent the effects of monocyte-derived DCs and/or CCL17-producing conventional DCs, but this has not been demonstrated directly. Perhaps too, resolving which DC subsets are most affected, which coreceptors are expressed, and their interaction with various T-cells including Treg, may help explain another study which reported a decrease in atherosclerosis from adoptive transfer of mature DCs pulsed with oxLDL (688). Yet, in another study, there was an increase in atherosclerosis with transfer of DC treated with MDA-LDL (769). Both studies used DCs expanded from bone marrow by incubation with GM-CSF. In another study, DCs were pulsed with both APOB-100 and IL-10 and then transferred into apoB-100 expressing LDLR KO mice. Treg were apparently activated, and atherosclerosis was reduced by a remarkable 70% (755).

Further complexity is illustrated by a study of DC engineered to have an extended lifespan. Prolonged survival of DCs was achieved by DC-specific transgenic expression of human Bcl-2 (595). Long-lived DC led to some signs of increased immunogenicity including greater Th1 activation (increased IFN-γ, IL-1β) and autoantibody production against oxLDL. However, there was also increased IL-10 and markedly greater expression of the coinhibitory receptor T-cell immunoglobulin 3 (TIM-3), typically seen on exhausted Th1, Th17, and CD8+ cells, but also seen on Treg (589). Yet, no change in Treg was seen in the mice with long-lived DC. There was a 20–25% decrease in total cholesterol to further complicate interpretation of the study. Overall, the effect on atherosclerosis was nil, with no change in lesion size or macrophage area (595).

2. Neutrophils may interact with plasmacytoid DCs to enhance atherosclerosis

Plasmacytoid DCs (pDCs) have recently attracted considerable attention. Besides expression of specific cell-surface markers, pDCs are distinguished by endosomal expression of TLR7 and TLR9. TLR7 responds to microbial RNA while TLR9 is triggered mainly by single-stranded viral or bacterial DNA. However, TLR9 can also respond to self-DNA if the DNA is complexed with cationic lipids or proteins (336, 981). Even greater pDC activation seems to occur in systemic lupus erythematosus when complexed DNA is presented to pDC TLR9 via FcγRII receptors which bind anti-DNA antibodies bound to DNA (980).

Activated pDC vigorously secrete type I interferons, especially IFN-α and IFN-β. Increased levels of type I interferons have been correlated with instability in human plaque (441). Repeated injections of IFN-α or IFN-β increased atherosclerosis in hyperlipidemic mice (see Table 7). In vitro, IFN-α stimulates vigorous production of IFN-γ and TRAIL (TNF-related apoptosis inducing ligand) by Th1 cells sufficient to kill VSMC. In addition, IFN-α amplifies innate immune responses by inducing upregulation of TLR4 on conventional DCs, as well as their production of TNF-α, IL-12, and IL-23 (441).

The TLR9 response is particularly of interest because of the potential for encounters with self-DNA and evidence that excess pDC stimulation and production of type I interferons are involved in autoimmune diseases such as systemic lupus erythematosus and psoriasis as well as in atherosclerosis. TLR9 responds quite specifically to nonmethylated, single-stranded viral or bacterial DNA oligomers containing cytidine-guanosine sequence motifs. These are referred to as “CpG” oligodeoxynucleotides (ODNs), where “p” refers to the phosphodiesterase bond between the deoxyribonucleotides. For example, one such CpG ODN used in a recent study was 5’-TCGTCGTTCCCCCCCCCCCCCCCCCCC-3’. The relevant CpG sequences are underlined. Various synthetic ODNs with bonds other than standard phosphodiester linkages are being studied as adjuvants for various vaccination protocols. Methylation of the cytosines greatly diminished but did not entirely eliminate responses of pDCs to CpG ODNs, but reversal of C and G (that is GpC instead of CpG) did essentially eliminate the TLR9 response (336). Importantly, TLR9 reactivity was greatly increased when CpG-containing ODN were complexed with the cationic
protein cathelicidin antimicrobial peptide (CAMP) (981). CAMP is the human protein; also known as LL37, it is referred to in mice as cathelicidin-related antimicrobial protein (CRAMP). Neutrophil granules contain large amounts of CAMP. CAMP also acts as a bactericidal protein on human skin where it is expressed by keratinocytes. CAMP and to a lesser extent other cationic proteins found in neutrophil granules bind the negatively charged phosphodiester backbone of DNA and protect it from rapid degradation by DNases normally found in the extracellular fluid (980). Nevertheless, specificity for nonmethylated CpG and the C-G order persists even after CAMP binding (336).

When incubated with CAMP, even human leukocyte DNA produced a substantial IFN-ß response in pDCs (980), but this response was only about one-sixth as great as bacterial DNA complexed with CAMP, since unmethylated CpG motifs are relatively infrequent in mammals (336).

Neutrophils are not only a source of CAMP (or CRAMP in mice), but also of extracellular DNA. Neutrophils undergo a specialized, pathogen-induced form of cell death (NETosis) that results in the ejection of a sticky meshwork of nuclear chromatin (including DNA, histones, and HMGB1) together with embedded cationic granule proteins including bactericidal permeability proteins, defensins, and large amounts of CAMP (209). These structures are called neutrophil extracellular traps (NETs) and are a major part of the neutrophil’s armamentarium against bacteria. Mast cells can also form NETs; and NET-like structures can be ejected from some intact cells by utilizing mitochondrial DNA (209).

NETosis seems to require input from simultaneous activating signals such as TLRs, Fc receptors, and cytokines such as IL-8, with subsequent PKC activation and production of superoxide by NADPH oxidase. NETs are not only bactericidal but provide both a scaffold and activation stimulus for intravascular thrombus formation (558, 559). Indeed, exposure to purified histones from disintegrated neutrophils was found to be sufficient for platelet stimulation. Interestingly, neutrophils from SLE patients seem predisposed to form NETs. Recently, NETs were found within and protruding from both mouse (439) and human (1179) atherosclerotic plaques. Higher plasma defensin levels were associated with greater risk of subsequent cardiovascular events and carotid intimal thickness progression (1279). Once partially activated macrophages enter the subendothelial space, they encounter trapped lipoproteins. Others have noted that pDC can promote Treg proliferation but also modulate Treg activity in a complex fashion (597, 1360). Possibly related was a doubling of atherosclerosis in response to DC-specific KO of the TGF-ß type II receptor which resulted in a decrease in pDC but enhanced proinflammatory DC signaling with increased TNF-α, IL-12, and markedly increased cytokine production by all the CD4+ subtypes (1043).

B. Lipoprotein Retention and Modification

Human autopsy specimens clearly show lipoprotein entry and retention in the intima preceding macrophage infiltration (1279). Once partially activated macrophages enter the subendothelial space, they encounter trapped lipoproteins. In classical in vitro studies, unmodified LDL, which binds essentially only the LDLR, did not cause foam cell formation due to LDLR downregulation once cellular cholesterol stores were replete. However, oxidized or acetylated LDL...
could promote foam cell formation as they were bound by various scavenger receptors that were not downregulated. Unmodified β-VLDL was found to induce foam cell formation without the need for further modification (115, 214). Since these studies, a number of additional LDL modifications, many without oxidation, have been shown to also suffice for foam cell formation (1727). VSMC underwent similar transformation into foam cells when presented with modified lipoproteins or β-VLDL.

1. LDL trapping by proteoglycans

To promote atherosclerosis, LDL and other lipoproteins must remain trapped in the intima long enough for modification and macrophage uptake. The importance of retention of LDL by binding to proteoglycans in the intimal extracellular matrix (particularly to those bearing chondroitin sulfate) was clearly demonstrated by the decrease in atherosclerosis seen in mice whose APOB lacked a proteoglycan binding determinant (1638). Other proteoglycan binding sequences appear to be present in APOB-48 as well (1732).

The corpulent rat (lacking the leptin receptor, LEPR due to a naturally occurring nonsense mutation) is hyperphagic, obese, and highly insulin resistant. It is unique among rat models in that animals develop atherosclerosis when fed a balanced-fat, high-cholesterol diet (1519). The atherosclerosis is unusual as there is abundant smooth cell proliferation together with foam cells and the rats develop intra-arterial thrombi in various vessels including the coronary arteries with resulting MI. Recently, cholesterol-fed corpulent rats were found to express a 2.8-fold increase in arterial wall biglycans and lengthened heparan sulfate chains. In addition, there was increased production of lipoproteins collected from intestinal lymph upon feeding that had an altered composition, being both larger and cholesterol enriched. In carotid arteries perfused ex vivo with test remnant lipoproteins, the corpulent rats’ arteries retained 2.5-fold more cholesterol. These differences were accompanied by a nearly fourfold increase in total ischemic myocardial lesions by 32 wk of cholesterol feeding (1133). Fasting plasma insulin strongly correlated with arterial wall biglycan. The investigators noted that biglycan production by VSMC is increased by fatty acids, ANG II, and TGF-β. Biglycan was also noted to be increased markedly in a porcine model of type I diabetes (1169). Biglycan is found together with LDL in human coronary arteries as well (1279). Increase in sulfation or glycosaminoglycan chain length, as found in this corpulent rat model, had previously been noted to cause greater lipoprotein retention.

Perlecan, the most abundant heparan sulfate glycosaminoglycan in the artery wall, also appears to promote LDL retention, at least acutely. It may also suppress VSMC proliferation (1805). Heterozygous deficiency of perlecan (1880) or excision of perlecan exon 3 (1805) reduced atherosclerosis significantly. In contrast, transgenic overexpression of decorin, a smaller proteoglycan, actually decreased atherosclerosis in ApoE-deficient mice (37). In this model, decorin production occurred primarily in the liver resulting in higher plasma decorin. Decorin was noted to bind and inhibit TGF-β, which can stimulate VSMC to synthesize larger proteoglycans that more effectively bind LDL (1055).

2. Lipoprotein modification by phospholipases

Atherosclerosis appears to be strongly promoted by certain secreted phospholipases (sPLA2), particularly groups III and V sPLA2 (1540). Group V sPLA2 seems to be the major sPLA2 enzyme and is released by activated endothelial cells (133, 134). Group IIa sPLA2 may also contribute but are less active than the group V enzyme (1981). LDL modified by group V sPLA2 aggregates binds more avidly to proteoglycans and is rapidly taken up into macrophages leading to foam cell formation. Accelerated atherosclerosis was seen in LDLR KO mice when group V sPLA2 was overexpressed. Reduced atherosclerosis was seen in mice with bone marrow transplants from mice deficient in group V sPLA2, suggesting macrophages or other leukocytes may also be a source of this enzyme (186). Interestingly, sphingomyelin inhibits group V sPLA2 and is found in greater concentration in ApoE KO lipoproteins, which were poor substrates for group V sPLA2. Furthermore, atherosclerosis in ApoE-deficient mice was not affected nearly as much by group V sPLA2 KO as it was in LDLR KO mice. Sphingomyelinase treatment further increased the activity of group V sPLA2 toward LDL. TGRL remnants that accumulate in ApoE KO mice may already be sufficiently modified or may not need any further modification to promote foam cell formation (198). Still, ApoE KO mice fed an atherogenic diet and overexpressing human group III sPLA2 had more than a twofold increase in atherosclerosis (1540).

In contrast to the above, transplantation of bone marrow deficient in the group X sPLA2 (PLA2G10) more than doubled atherosclerosis in the aortic sinus of LDLR-null mice. At the same time, there was a decrease in the number of macrophages but a marked increase in macrophage necrosis and a fourfold increase in necrotic core size. Transgenic overexpression of human PLA2G10 reduced plaque size 50%. Enhanced Th1 response with increased IFN-γ was seen with PLA2G10 deficiency and suppressed by transgenic overexpression while Th2 and macrophage IL-10 production was increased by PLA2G10 overexpression (29). The authors suggested that the nonspecific inhibition of all sPLA2 enzymes by varespladib may have been responsible for the premature ending of a recent phase III trial for futility.

3. Electronegative LDL and secretory acid sphingomyelinase

Secretory acid sphingomyelinase and lysosomal acid sphingomyelinase are derived from the same gene with different posttranslational processing. Secretory acid sphingomyeli-
nase is released by activated endothelial cells and other lesional cells and has been implicated in LDL modification. Importantly, sphingomyelinase produces ceramide in the lipoprotein which promotes greater retention by proteoglycans and greatly promotes uptake and foam cell formation for several lipoprotein types. Deficiency of acid sphingomyelinase in LDLR KO mice resulted in a 55% decrease in atherosclerosis (417). Not only might sphingomyelinase be important within the subendothelial space, but a fraction of plasma LDL appears to carry substantial sphingomyelinase activity. These LDL were also electronegative and carried PAF-AH (also referred to as Lp-PLA2). Electronegative LDL, when incubated in vitro, were shown to degrade their own phospholipids, rendering them highly subject to aggregation and macrophage uptake. Such LDL could also activate inflammatory responses from various cell types (103, 1535).

4. Oxidatively modified lipoproteins

Much has been written regarding oxidative modification of lipoproteins. Certainly when oxidized by various methods, including incubation with cultured endothelial cells (which are generally at least partially activated in most in vitro settings), LDL and other lipoproteins are taken up avidly by macrophages with resultant macrophage activation, proliferation, and foam cell formation (1682). Some of these effects are likely mediated by oxidation of phospholipids (131). Oxidation of cholesteryl esters (721), changes to APOB characterized by chlorination of tyrosine by myeloperoxidase, tyrosine cross-linking, and oxidative nitration may be important as well (1304). Furthermore, oxidation of HDL clearly impairs its antithrombotic function (1304). These and many other observations make a persuasive argument that oxidation of lipoproteins contributes to atherosclerosis (739, 1682).

Despite mechanistic support for oxidation in atherosclerosis, human clinical trials with antioxidants have been resoundingly negative (particularly the larger and more recent ones) (155, 1573, 1595). A possible reason for this apparent discrepancy may be, once again, redundancy. Here, the redundancy involves multiple possible lipoprotein modifications that could all lead to foam cell formation. One additional consideration may be important. The chemical oxidative footprint found predominantly on LDL from human atherosclerotic plaque clearly points to myeloperoxidase as the source of pro-oxidant activity (740). This would suggest that activated macrophages and possibly neutrophils are the main cause of such oxidized LDL. This brings into question the primacy of oxiLDL (or even mmLDL) in activating the macrophage in anticipation of foam cell formation, since activated macrophages or neutrophils would have to already be present in the lesion to initiate such oxidation. While oxiLDL generated by myeloperoxidase would undoubtedly be atherogenic and promote further foam cell formation, as well as inflammation and apoptosis, it would seem unlikely to be an initial cause of macrophage activation (and perhaps not even a necessary one). Furthermore, the LDL could be oxidized after it was taken up into the macrophage and only become available for (re)uptake after apoptosis or necrosis of the primary phagocyte. Unfortunately, standard mouse models of atherosclerosis are not of much help in resolving the issue of myeloperoxidase involvement as mice express much lower levels of myeloperoxidase in their macrophages than seen in human lesions. Furthermore, knockout of mouse myeloperoxidase does not decrease atherosclerosis. However, overexpression of human myeloperoxidase in the mouse macrophages does increase atherosclerosis (1304). These considerations support the notion that myeloperoxidase is potentially proatherogenic but redundant, as mouse atherosclerosis can progress extensively in its absence.

5. Other chemical modification of LDL

Carbamylation is a recently recognized form of LDL modification that may be particularly important in renal disease and smoking. A small fraction of urea can spontaneously and reversibly form highly reactive cyanate, which can then bind to lysine residues in proteins forming homocitrulline. Many proteins are altered in uremia, but carbamylated LDL from uremic patients or prepared in vitro were found to activate endothelial cells primarily by way of LOX-1 (70–72). Furthermore, carbamylated LDL binds macrophage SR-AI receptors and causes foam cell formation (1926). Importantly, thiocyanate, which is markedly increased in the plasma of cigarette smokers, can be converted to cyanate by myeloperoxidase, also leading to carbamylation. Protein homocitrulline levels were found strongly related to cardiovascular risk with an odds ratio of 7–8 in the top versus bottom quartile (1926). Indoxyl sulfate is another uremic toxin that can modify LDL which then may cause endothelial activation and inflammation (832).

C. Uptake of Modified Lipoproteins and Foam Cell Formation

1. Bridging molecules facilitate lipoprotein uptake

Lipoprotein lipase (LPL) secreted by macrophages can provide a nonenzymatic bridge between lipoproteins and proteoglycans and promote retention in the arterial wall and increased atherosclerosis (as shown by bone marrow transplant studies with LPL deficient macrophages) (94). LPL may also promote uptake into macrophages. Conversely, endothelial cell LPL is protective, consistent with more active removal of triglyceride-rich lipoproteins and increased HDL (1624). ANGPTL3 and ANGPTL4, which can inhibit endothelial LPL, were proatherogenic in hyperlipidemic mice. Hepatic LPL also appears to be protective since KO of Fhx2, a corepressor of LPL production in the liver, was protective (see TABLE 8).
Endothelial lipase (EL, also known as LIPG for lipase G), while usually associated with remodeling and reductions of plasma HDL can also serve as a bridge to bind LDL to cell heparan sulfated proteoglycans, promoting uptake of both native and oxLDL followed by cholesterol accumulation in macrophages (1444). EL KO in ApoE null mice reduced atherosclerosis 70% (828). Human ApoE4 expressed in LDLR KO mice promoted uptake of LDL into macrophages and increased atherosclerosis. Secretion by macrophages and VSMC of HSP27 was protective by apparently blocking uptake of modified lipoproteins via scavenger receptor A (SR-A) (see Table 8).

2. Scavenger receptors take up modified lipoproteins and β-VLDL

Oxidatively modified LDL as well as some other modified LDL and β-VLDL are taken up by a variety of scavenger receptors (654, 1422). These include the class A receptors (AI, AII, MARCO, SRCL, SCARA5), class B receptors (CD36, SR-B1), class C (dSR-C1; in Drosophila only), class D (CD68; an endosomal receptor), class E (LOX-1, others), class F (SREC-I, SREC-II), class G (SR-PSOX; also known as CXCL16), class H (FEEL-1, FEEL-2; primarily bacterial binding), and class I receptors (CD163; which binds hemooglobin/haptoglobin complexes). A number of these receptors bind acetylated LDL and especially oxLDL (SR-AI/II, MARCO, SRCL, SR-B1, LOX-1, SREC-I, FEEL-1/2). CD36 also binds VLDL and TGRL remnants. LOX-1 was discussed previously in the context of endothelial activation but also serves to promote foam cell formation. Of the other receptors, only SR-A and CD36 have been studied extensively. Importantly, all these scavenger receptors bind alternative ligands which may reflect their physiological roles. Thus scavenger receptors bind various bacteria, bacterial LPS, lipoteichoic acid (LTA), microbial diglycerides, apoptotic debris, amyloid, and AGEs. Macrophages from mice deficient in SR-AII bound AGE much less avidly. Also, the mice were more prone to death from infection with Listeria and particularly with HSV-1 infections (1718). Thus macrophage scavenger receptors play a major role in recognizing PAMPs and hence participating in innate immunity. Later it was shown that SR-A is the main scavenger receptor for group B Streptococcus and Streptococcus pyogenes but could also bind Neisseria meningitidis, Staphylococcus aureus, and E. coli (654). Binding of CD36 can trigger inflammatory activation of NF-κB and MAPK cascade signaling by interactions with Src kinases (1230). Nevertheless, scavenger receptors can also play an important anti-inflammatory role in clearing apoptotic debris. Genetic manipulation of these and other promoting pathways are reviewed in Table 8.

Results from studies deleting scavenger receptors have been quite varied. Some investigators reported reduced atherosclerosis upon deletion of the macrophage SR-A (1530, 1718) while others did not (654). CD36 knockout seemed to achieve greater atherosclerosis reductions (45–74%) (507, 958) while combined absence of SR-A and CD36 was similar to CD36 deficiency alone (958). Yet, in another study in ApoE KO mice fed a high-fat diet for just 8 wk, either CD36 or SR-A deficiency modestly increased plaque sizes in the aortic sinus while aortic plaque area decreased (1231). This was observed despite 80–90% reductions of in vitro oxLDL uptake and foam cell formation by macrophages lacking both SR-A and CD36 previously seen by the same group. Differences in the length of the studies may have contributed to the differences in study outcomes, but ultimately, the discrepancies remain unexplained. It should be mentioned that CD36 appears to act as a long-chain fatty acid transporter in muscle, heart, and adipose to facilitate uptake of fatty acids. In small intestine it facilitates chylomicron synthesis (629). Recently, intraperitoneal administration of a small molecule inhibitor of CD36 in LDLR and leptin doubly deficient mice was reported to reduce serum triglyceride levels by 50% and decrease atherosclerosis to a similar extent (602). These studies suggest that effects of CD36 KO may be more complex than originally envisioned and difficult to predict.

In addition to traditionally cited scavenger receptors as mediators of modified lipoprotein uptake, recent evidence points to non-scavenger receptor uptake as potentially a quantitatively more important pathway. LDL treated with group V sPLA$_2$ is taken up by macropinocytosis which is mediated by syndecan 4. This process is accelerated by macrophage activation and can lead to foam cell formation (318). Unbound native LDL can also be taken up in substantial quantities by this process. In general, macropinocytosis is upregulated by pro-inflammatory signaling, such as TLR4 and Syk activation which can be mediated by oxLDL, acetylated LDL, mmLDL, LDL treated by 15-LO, PMA activation of PKCβ and PKCδ, cholesteryl ester hydroperoxides, oxysterol-binding protein-related protein 2 (ORP2), and even viruses. In contrast, LXR agonists, which are anti-inflammatory, block macrophage fluid-phase uptake of LDL. Unlike uptake of oxLDL through scavenger receptors, macropinocytosis is blocked by inhibition of PI3Kγ (956). Thus foam cells can clearly be formed independent of scavenger receptors. In a sense then, scavenger receptor uptake of modified LDL may be considered yet another example of redundant pathways.

D. Intracellular Cholesterol Homeostasis in Macrophages

1. Extracellular peptides and intracellular signaling affecting scavenger receptor expression, lipoprotein uptake, and cholesterol efflux

Part of the activation repertoire of macrophages, and probably VSMC, is an increase in surface expression of scavenger receptors together with greater uptake of mod-
ifed lipoproteins and enhanced activity of ACAT to es-
terify free cholesterol derived from engulfed lipoproteins.
Several classically proatherosclerotic cytokines including
IFN-γ, TNF-α, and IL-1β promote foam cell formation in this manner. They also contribute to enhanced foam cell
formation by downregulation of cholesterol efflux (1174).
To this list of well-known proinflammatory cyto-
kines was added the TNF-family cytokine
TNF-like protein 1A (TL1A) which binds to its receptor
death receptor 3 (DR3). Activation of DR3 signaling in
macrophages results in increased uptake of acetylated
LDL (AcLDL) and oxLDL, presumably by the observed
upregulation of SR-A, CD36, SR-B1, and LPL. Simulta-
neously, cholesterol efflux was diminished together with
suppressed secretion of ApoE and downregulation of
ABCA1 and ABCG1 transporters (1173). This coordi-
nated induction of scavenger pathways together with
downregulation of cholesterol efflux pathways has be-
come apparent in a number of studies. Opposed to these
effects (with essentially mirror image activities) are cyto-
kines which block foam cell formation and favor choles-
terol efflux. These include TGF-β and IL-10 (1174) to
some extent, but more specifically IL-33 binding to its
receptor ST2L on macrophages (1175). Elevation of mac-
rophage cAMP may also be part of this response as glu-
cagon-like peptide 1 (GLP-1) binding to its Gaαi-coupled
receptor GLP1R as well as gastric inhibitory peptide
(GIP) with its Gaαi GPCR both decreased foam cell for-
mation and atherosclerosis during prolonged administra-
ion (1271). The peptide salusin-α also decreased foam
cell formation and atherosclerosis while salusin-β had
opposing effects (see TABLE 8).

One of the main controllers of macrophage cholesterol
homeostasis is the nuclear receptor LXR which combines
as a heterodimer with retinoid X receptor (RXR) to func-
tion as a transcription factor. Pharmacological activation of
LXR inhibits atherosclerosis while KO results in in-
creased atherosclerosis (243, 1618, 1756). Roughly in
proportion to its levels, intracellular cholesterol is spon-
taneously or enzymatically (by CYP27) oxidized to ox-
yesters (such as 25- or 27-hydroxycholesterol). These
bind to and activate LXR. At the same time, all the PPAR
nuclear receptors bind various fatty acids and oxidized
fatty acid derivatives taken up with modified LDL, other
lipoproteins, and phagocytosed apoptotic cells. These
oxidized fatty acids, or possibly some unchanged fatty
acids, act as endogenous PPAR ligands. Some of these
pathways are illustrated in FIGURES 16 AND 17. Active
PPARγ specifically induces LXRα as well as CYP27, thus
amplifying the LXR response. When bound to ligand,
LXRα dimerizes with RXR and increases transcription of
a number of genes involved with cellular cholesterol bal-
ance including NPC1/2 (Niemann-Pick type C 1 and 2
which are involved in cholesterol intracellular traffick-
ing), ABCA1, ABCG1, ApoE, phospholipid transfer pro-
tein (PLTP), SCD-1, LPL, Mertk, IRF8, and SREBP-1c.
All of these genes (with the exception of SREBP-1c and
LPL) either facilitate cholesterol efflux or are otherwise
protective against atherosclerosis (see TABLE 8). IRF8 is a
transcription factor that increases expression of protec-
tive Arg1 (arginase 1) that helps form inflammation-res-
olving polyamines (see TABLE 8). Overexpression of
LXRα increased net cholesterol transport from macro-
phages to the intestine, promoted intestinal non-bile
transintestinal cholesterol excretion (TICE), and reduced
liver fat while reducing atherosclerosis by 41% (1066).
Of note, LXRβ was not protective apparently because
when it is not bound by agonist it represses protective
ABCA1 and AGCG1, although it also decreases SREBP-1
expression (152).

Activation of PPARγ also upregulates CD36 expression
(287), leading to both increased uptake of lipid through
CD36 but also increased LXR-mediated efflux of choles-
terol to HDL or other acceptors, with an overall increase in
net cholesterol flux and reverse cholesterol transport. KO of
PPARγ in hematopoietic cells modestly increased athero-
sclerosis (287) while deletion of its coactivator, PGC-1α,
had a neutral effect (1679). Adipocyte enhancer-binding
protein 1 (AEBP1) is a transcriptional repressor that down-
regulates LXR and PPARγ activity in macrophages. AEBP1
overexpression in a BMT study resulted in greatly accel-
ereated atherosclerosis in ApoE null mice (1116). Macro-
phages from AEBP1 transgenic mice showed reduced LXR,
PPARγ, ABCA1, ABCG1, and 1xB with increased translo-
cation of NF-κB to the nucleus followed by increased IL-6
and TNF-α expression.

Nrf2, although seemingly highly protective in endothelial
cells, supports expression of CD36 and foam cell formation
in macrophages. Thus atherosclerosis was reduced in fat-
fed or chow-fed hyperlipidemic mice with Nrf2 KO (106,
1717). The recent finding that Nrf2 supports NLRP3 in-
flamasome formation (546) (see below) provides further
insight into the role of Nrf2 in atherosclerosis.

ABCB4 (also known as P glycoprotein 3) transports phos-
pholipids into bile, thereby facilitating biliary cholesterol
secretion, but is also present on macrophages. Foam cell
formation in ABCB4-deficient macrophages was greatly ac-
celerated upon incubation with acetylated LDL due to in-
creased activity of scavenger receptors. Neither cholesterol
nor phospholipid efflux to HDL was affected (1403). These
findings raise the question of whether other cholesterol and
phospholipid acceptors might be equally important as HDL
in macrophage cholesterol homeostasis.

Finally, despite less inflammatory signaling, RIP2 deficiency
increased atherosclerosis apparently because RIP2-deficient
macrophages took up more lipid and increased foam cell
formation due to constitutively active TLR4/Syk signaling
which promoted macrophage spreading and macropinocytosis (1012).

2. Intracellular lipid trafficking in macrophages and foam cells

As macrophages take up modified LDL and TGRL remnants, cholesteryl ester in lysosomes is hydrolyzed by lysosomal acid lipase A (LIPA). Deficiency of LIPA promotes atherosclerosis, possibly due to lysosomal damage and necrosis caused by excess lipid accumulation (448, 2124). The action of normal LIPA releases free cholesterol which is transported between cell organelles by NPC1/2. ApoE-deficient mice with NPC1 KO have worse atherosclerosis, and interestingly, severe, spontaneous thrombosis. The cause of the pro-thrombotic state was not clear (1940). Released free cholesterol is esterified by acyl-CoA:cholesterol acyltransferase 1 (ACAT1) and stored in intracellular lipid droplets. Cholesteryl ester (CE) droplets contribute to foam cell formation, and ultimately the size of plaques but appear to be less problematic than excess free cholesterol which is cytotoxic. Accordingly, bone marrow deficiency of ACAT1 in hyperlipidemic mice caused a marked increase in atherosclerosis (see TABLE 8), presumably due to accumulation of free cholesterol. Nevertheless, deletion of the enzyme Elovl6 (elongation of very long-chain fatty acids family member 6) from macrophages in LDLR-null mice decreased foam cell formation and atherosclerosis (1529). Elovl6 (elongation of very long-chain fatty acids family member 6) from macrophages in LDLR-null mice decreased foam cell formation and atherosclerosis (1529). Elovl6 (elongation of very long-chain fatty acids family member 6) from macrophages in LDLR-null mice decreased foam cell formation and atherosclerosis (1529). Elovl6 (elongation of very long-chain fatty acids family member 6) from macrophages in LDLR-null mice decreased foam cell formation and atherosclerosis (1529). Elovl6 (elongation of very long-chain fatty acids family member 6) from macrophages in LDLR-null mice decreased foam cell formation and atherosclerosis (1529). Elovl6 (elongation of very long-chain fatty acids family member 6) from macrophages in LDLR-null mice decreased foam cell formation and atherosclerosis (1529). Elovl6 (elongation of very long-chain fatty acids family member 6) from macrophages in LDLR-null mice decreased foam cell formation and atherosclerosis (1529). Elovl6 (elongation of very long-chain fatty acids family member 6) from macrophages in LDLR-null mice decreased foam cell formation and atherosclerosis (1529). Elovl6 (elongation of very long-chain fatty acids family member 6) from macrophages in LDLR-null mice decreased foam cell formation and atherosclerosis (1529). Elovl6 (elongation of very long-chain fatty acids family member 6) from macrophages in LDLR-null mice decreased foam cell formation and atherosclerosis (1529). Elovl6 (elongation of very long-chain fatty acids family member 6) from macrophages in LDLR-null mice decreased foam cell formation and atherosclerosis (1529). Elovl6 (elongation of very long-chain fatty acids family member 6) from macrophages in LDLR-null mice decreased foam cell formation and atherosclerosis (1529). Elovl6 (elongation of very long-chain fatty acids family member 6) from macrophages in LDLR-null mice decreased foam cell formation and atherosclerosis (1529). Elovl6 (elongation of very long-chain fatty acids family member 6) from macrophages in LDLR-null mice decreased foam cell formation and atherosclerosis (1529). Elovl6 (elongation of very long-chain fatty acids family member 6) from macrophages in LDLR-null mice decreased foam cell formation and atherosclerosis (1529). Elovl6 (elongation of very long-chain fatty acids family member 6) from macrophages in LDLR-null mice decreased foam cell formation and atherosclerosis (1529). Elovl6 (elongation of very long-chain fatty acids family member 6) from macrophages in LDLR-null mice decreased foam cell formation and atherosclerosis (1529). Elovl6 (elongation of very long-chain fatty acids family member 6) from macrophages in LDLR-null mice decreased foam cell formation and atherosclerosis (1529). Elovl6 (elongation of very long-chain fatty acids family member 6) from macrophages in LDLR-null mice decreased foam cell formation and atherosclerosis (1529). Elovl6 (elongation of very long-chain fatty acids family member 6) from macrophages in LDLR-null mice decreased foam cell formation and atherosclerosis (1529). Elovl6 (elongation of very long-chain fatty acids family member 6) from macrophages in LDLR-null mice decreased foam cell formation and atherosclerosis (1529). Elovl6 (elongation of very long-chain fatty acids family member 6) from macrophages in LDLR-null mice decreased foam cell formation and atherosclerosis (1529). Elovl6 (elongation of very long-chain fatty acids family member 6) from macrophages in LDLR-null mice decreased foam cell formation and atherosclerosis (1529). Elovl6 (elongation of very long-chain fatty acids family member 6) from macrophages in LDLR-null mice decreased foam cell formation and atherosclerosis (1529).

TABLE 8

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIPA</td>
<td>Hydrolyzes cholesteryl ester</td>
</tr>
<tr>
<td>ACAT1</td>
<td>Esterifies free cholesterol</td>
</tr>
<tr>
<td>NPC1/2</td>
<td>Transport between cell organelles</td>
</tr>
<tr>
<td>Elovl6</td>
<td>Decreases foam cell formation</td>
</tr>
</tbody>
</table>

Macrophage lipid droplets are surrounded by several proteins including perilipin 1, ADRP (also known as perilipin 2 or PLIN2), TIP47 (tail-interacting protein of 47 kDa), and S3–12 (1397). Cholesteryl ester stored in these droplets can be hydrolyzed by several enzymes with neutral cholesteryl ester hydrolase activity. These enzymes include neutral cholesteryl ester hydrolase 1 (NCEH1), cholesteryl ester hydrolase (CEH), and hormone-sensitive lipase (HSL); interestingly, all three were found to be protective against atherosclerosis (see TABLE 8). Free cholesterol released by these enzymes appears to be efficiently removed from macrophages through ABCA1 and AGCG1 transporters (1908).

The recently identified niacin receptor GPR109A is a G<sub>i</sub> type GPCR expressed on the surface of macrophages, other white blood cells, and adipocytes but not endothelial cells. One physiological function in adipocyte is the suppression of free fatty acid mobilization and stimulation of adiponecin release when GPR109A is bound by the endogenous ligand β-hydroxybutyrate, a ketone body (1419). Interestingly, the antiatherogenic effects of niacin in LDLR null mice were entirely dependent on bone marrow expression of GPR109A and were present despite a lack of effect on serum lipids (1087).

E. Macrophage Cholesterol Efflux and Atherosclerosis

Because excessive free cholesterol is toxic to cells, several means to deal with this substance are available. ABCA1 is a large protein with multiple transmembrane domains that forms homotetramers or higher-order oligomers and facilitates free cholesterol and phospholipid efflux to lipid-poor apo A-1 (often referred to as pre-β HDL for its electrophoretic mobility). The more recently identified ABCG1 functions as a homodimer and can promote cholesterol and oxysterol efflux to larger, spherical HDL<sub>2</sub> and HDL<sub>3</sub> particles. Further details regarding the mechanism of efflux to HDL and related signaling will be provided in the section on HDL below (see sect. XE). APOA1 KO clearly increased atherosclerosis (see TABLE 8). In contrast, overexpression of APOA2, another HDL apoprotein, increased atherosclerosis in a particular mouse model that may not be relevant to humans (see TABLE 8). APOA1 is generally regarded as primarily produced in liver, but KO of the transcription factor retinoic acid receptor-related orphan receptor α (RORα) resulted in markedly increased atherosclerosis and a profound reduction in HDL due to reduction of APOA1 production in intestine, not liver (1132).

Both ABCA1 and ABCG1 appear to be important for macrophage cholesterol efflux and prevention of inflammatory and cytotoxic effects of macrophage cholesterol overload (1447, 1749). ABCA1 and ABCG1 expression are induced by LXR. ABCG1 can also be induced directly by PPARγ. Activation of AMPK caused posttranslational stabilization of ABCG1 mRNA and prevented foam cell formation. Atherosclerosis was decreased by oral administration of AICAR, an analog of AMP that activates AMPK (1021). IFN-γ and ANG II downregulate ABCA1 and promote foam cell formation (1884).

ABCG4 can also be induced by LXR and promote cholesterol efflux from macrophages but appears to be most important in brain. ABCA5 can promote cholesterol efflux to HDL and may also be protective against atherosclerosis while ABCA7 had no effect on atherosclerosis (see TABLE 8). Net transfer of cholesterol to HDL through macrophage SR-B1 appears minimal in mice, so its antiatherogenic effect is less likely to involve cholesterol removal from foam cells in mouse models (1908). SR-B1 can mediate protective intracellular signaling in endothelial cells after binding HDL, and this requires the adapter PDZK1 binding to the intracellular domain of SR-B1. KO of either SR-B1 or PDZK1
increased atherosclerosis (see TABLE 8). These signaling effects of HDL are reviewed further in section XE. Macrophages also constitutively secrete ApoE which can act together with phospholipid as a free-cholesterol acceptor, forming disks much like nascent HDL (2082). ApoE also acts in efferocytosis as reviewed below.

ABCA1 and ABCG1 (and probably ABCA5) show compensation for one another’s deficiency while macrophage-specific knockout of both ABCA1 and ABCG1 clearly increased atherosclerosis (2054). These insights help explain variable results seen with knockout of only ABCA1 or ABCG1 (22). Hepatic ABCA1 expression may be most important in atherogenesis through preservation of newly secreted APOA1. This may be followed by rapid minimal lipidation of pre-β HDL before leaving the liver, perhaps in the space of Disse (220, 1249). Without such lipidation of nascent particles, lipid-poor APOA1 is rapidly eliminated by the kidneys and HDL levels are extremely low, as in Tangier disease patients (who lack ABCA1).

Surprisingly, HL KO decreased atherosclerosis in ApoE-deficient mice (1204). HL is most active toward intermediate-sized lipoproteins, hydrolyzing both TG and phospholipid in larger HDL and in IDL (intermediate density lipoproteins). Even though HL−/−Apoe−/− mice had much higher total cholesterol and β-VLDL, they also had higher HDL-C. Furthermore, HDL from ApoE−/− are severely impaired in promoting eflux from cultured cells and HL deficiency partially restored this capacity.

**F. Whole-Body Reverse Cholesterol Transport and Atherosclerosis**

Assessment of in vivo whole-body reverse cholesterol transport in mice involves injecting macrophages loaded with radiolabeled cholesterol into the mouse peritoneum and tracking appearance of the cholesterol label in blood and feces (400, 1447). This methodology may provide insights into the role of reverse cholesterol transport in atherogenesis and may help explain a number of seemingly paradoxical findings. Thus whole-body or hepatic SR-B1 deficiency increased HDL but decreased reverse cholesterol transport through HDL and increased atherosclerosis. Conversely, overexpression of hepatic SR-B1 lowered HDL but increased reverse cholesterol transport and inhibited atherosclerosis (400).

The enzyme lecithin-cholesterol acyl transferase (LCAT) esterifies free cholesterol in HDL in the plasma space and is often considered a means of increasing the capacity of HDL to accept more free cholesterol. Surprisingly, however, LCAT overexpression increased atherosclerosis. Very high LCAT actually impaired whole body reverse cholesterol transport by decreasing availability of pre-β HDL. Conversely, LCAT KO may decrease atherosclerosis in some mouse models. Furthermore, in LCAT-overexpressing mice, transgenic expression of cholesterol-ester transport protein (CETP) appears to facilitate reverse cholesterol transport and decrease atherosclerosis even though plasma HDL-C is decreased (1447). In other studies, CETP expression in mice promoted reverse cholesterol transport with a requirement for a functional LDLR for final delivery of cholesterol to the liver (1757).

Another interesting example of potential complexities in reverse cholesterol transport is the role of PLTP (364, 1350). PLTP is found in plasma associated with HDL and mediates complex HDL remodeling. Excess surface phospholipid and unesterified cholesterol that remain on TGRL remnants after delipidation (as well as some from LDL) are transferred by PLTP to HDL. In addition, PLTP causes fusion of smaller HDL3, yielding both larger HDL2 and smaller spherical HDL, pre-β HDL, or free APOA1. ApoM may also interact with PLTP in regeneration of pre-β particles. High plasma or “systemic” PLTP activity appears generally atherogenic, perhaps because increased generation of pre-β HDL or free APOA1 in plasma would cause greater renal loss of APOA1, resulting in low HDL and decreased overall APOA1 availability. Whole-body PLTP deficiency in two models of hyperlipidemic mice led to reduced production of VLDL and LDL and marked reduction of serum lipids, together with reduced atherosclerosis (852). Lower systemic PLTP activity predicted by a genetic score in humans was also associated with reduced coronary risk (975). In contrast, PLTP released locally by macrophages appears protective since PLTP physically interacts with ABCA1 and pre-β HDL to promote cholesterol eflux (1350). Furthermore, PLTP interaction with ABCA1, even when the lipid transfer activity is eliminated by a PLTP M159E mutation, stimulates JAK2 and anti-inflammatory STAT3 signaling in macrophages (1891). Thus whole-body KO of PLTP in LDLR−/− mice was atheroprotective (presumably by preserving APOA1 generally as discussed above). However, transplant of bone marrow from PLTP+/+ LDLR−/− mice into PLTP−/− LDLR−/− mice further reduced atherosclerosis, demonstrating the protective role for normal local expression of PLTP by macrophages (1839). This model resolves a number of seemingly discrepant observations regarding PLTP (364, 1840, 1856). Interestingly, PLTP also binds and inactivates bacterial LPS, and PLTP-deficient mice tolerate endotoxemia poorly. The anti-inflammatory effect of PLTP may also be important in regard to multiple sclerosis, as lower PLTP activity was associated with more active disease (1891).

**X. PROGRESSION OF ATHEROSCLEROSIS**

**A. Features of Unstable Plaques: Challenges With Animal Models**

As noted in the introduction, this phase of atherogenesis has to do with growth of complex plaques, plaque rupture, and subsequent thrombosis with precipitation of acute ischemic
events such as MI. While animal models of these processes still have limitations, much progress has been made (837, 1300, 1576). Several models of complex plaque have been developed and are being used in contemporary studies (see TABLE 9). High-fat feeding of ApoE-deficient mice leads to plaque ruptures in the brachiocephalic artery (857). A related but less widely recognized or utilized model is the ApoE2 knock-in mouse which closely mimicks human type III hyperlipidemia. These mice develop lesions with fibrous caps, necrotic cores, and cholesterol clefts (1703). LDLR-deficient mice can be additionally engineered to only express APOB-100 by deletion of APOB editing catalytic polypeptide 1 (APOBEC1) causing absence of APOB-48 expression. These mice have marked LDL elevations without requiring additional high-cholesterol, high-saturated fat diets and develop severe, complex lesions (1121, 1534). Mice with combined LDLR and ApoE deficiency suffer extremely accelerated atherosclerosis and experience spontaneous MI which may be related to endothelin signaling as a precipitating event (242). Akt1 (PKB) deficiency in ApoE-null mice results in accelerated atherosclerosis with pronounced VSMC and macrophage apoptosis, friable fibrous caps, and spontaneous MI (520).

Silastic cuff placement around the carotid artery accelerates atherosclerosis (1886). Ligation followed by cuff placement on a carotid artery results in stenosis, rupture, and thrombosis in ApoE-deficient mice (1539). Endothelial activation by disrupted flow likely accelerates atherogenesis and susceptibility to thrombosis in these models (1299, 1301). Hyperlipidemic SR-B1 and PDZK1 KO mice develop coronary stenosis and MI (2034). Coronary occlusion occurred frequently in ApoE KO mice lacking CD59, an inhibitor of later stages of complement activation and formation of the membrane attack complex (1985). Fibrillin-1 (FBN1) haploinsufficiency leads to plaque instability and rupture (1857). Coronary stenosis and MI was frequent in ApoE-deficient mice overexpressing urokinase-type plasminogen activator (uPA) in macrophages (354). Effects on atherosclerosis of these and other studies, frequently using a combination of interventions or double KO models, are reviewed in TABLES 9 AND 10 and will be reviewed in more detail below.

In human pathological studies, vulnerable plaque is characterized by greater cholesterol content, greater numbers of activated macrophages and foam cells especially in the shoulder area, increased markers of apoptosis, a thin fibrous cap, decreased collagen content, and more inflammatory activity including matrix metalloproteinase (MMP) expression. These features lead to rupture or fissuring in the fibrous cap in ~60–75% of cases of sudden death in humans (935). There is exposure of the highly thrombogenic plaque substance to blood and precipitation of acute thrombotic events (351, 386, 1882). Approximately 25–40% of acute coronary events in humans can be attributed to thrombus formation on erosions (areas of endothelial cell loss but without rupture) (935). More recently, markers of red blood cell infiltration, hypoxia with release of hypoxia-inducible factors (HIF), and plaque penetration with vaso vasorum have been related to enlargement of lesions and instability (1162, 1644, 1826, 1882).

Abrupt crystallization of free cholesterol has recently been proposed as the immediate precipitant for most acute obstructive events (4). Distinct volume expansion occurs with cholesterol crystallization, possibly providing the underlying force to precipitate fissures or frank rupture of the fibrous cap (3, 1870). Typical tissue preparation techniques use ethanol for fixation which dissolves cholesterol crystals and eliminates their visualization. When pathological specimens were air dried, virtually all lesions associated with acute coronary events were seen to have cholesterol crystals penetrating through to the vessel lumen while none were found in autopsy controls (4).

B. Macrophage and Smooth Muscle Cell Trafficking and Proliferation

In a very basic sense, in order for plaques to increase in size, become complex, and threaten acute coronary events, macrophages and/or VSMC must either continue to move into the growing plaque or proliferate or both. These fundamental issues will be examined in this section.

1. Macrophage trafficking in atherosclerotic lesions

High lipid content in human plaques is one of the strongest correlates of instability (511). All measures of plaque vulnerability including macrophage content and activation, indices of apoptosis, MMP activity, and collagen content are improved rapidly in animal studies when plasma lipid levels are reduced by change in diet (24, 26, 27, 927) or by statins (25, 566, 1532).

Extensive egress of foam cells was seen within days of transplanting atherosclerotic vessels from genetically hyperlipidemic mice into wild-type mice (1810, 1962). This egress was dependent on signaling in foam cells through the CCR7 chemokine receptor and could be blocked by antibodies against the natural ligands to CCR7, namely, CCL19 and CCL21. Furthermore, expression of CCR7 was increased by LXR agonists and greatly impaired by genetic LXR deficiency (508).

Despite these fascinating results from transplant studies, more recent studies with quantitative assessment of macrophage entry and egress rates employing monocytes labeled with latex beads revealed that macrophage recruitment and entry into the plaque is by far the most important determinant of the number of macrophages in a plaque (1434). These studies were performed using a...
### Table 9. Genes related to further plaque growth, progression of advanced atherosclerosis (54 genes tested)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Model</th>
<th>%ΔA</th>
<th>Function, Comment</th>
</tr>
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<tbody>
<tr>
<td>ATM</td>
<td>ATM−/− apoE−/−</td>
<td>244%</td>
<td>Whole body ATM KO mice also had increased body fat, higher blood pressure, glucose intolerance, and insulin resistance. ATM−/− apoE−/− had intermediate atherosclerosis. BMT resulted in 80% increased atherosclerosis but with no metabolic effects (1561). Similar results reported by another group (1195).</td>
</tr>
<tr>
<td>p53</td>
<td>p53−/− apoE−/−</td>
<td>101%</td>
<td>Increased macrophage proliferation was seen in p53 KO, not increased apoptosis (663). Similar results with apoE Leiden mice (1682). Others report normal p53 expression in atherosclerotic plaques limits plaque size by promoting apoptosis of macrophages while protecting VSMC and stromal cells from apoptotic death (1194).</td>
</tr>
<tr>
<td>Rb</td>
<td>Cre-lox macrophage p53−/− in apoE−/−</td>
<td>NS</td>
<td>No change in lesion size but less macrophage apoptosis and fewer cholesterol crystals in plaques (164).</td>
</tr>
<tr>
<td>Kip1 (CDKN1B)</td>
<td>Kip1−/− apoE−/−</td>
<td>483%</td>
<td>Kip1 (CDK interacting protein also known as p27 or p27Kip1) is a cell-cycle inhibitor by inhibiting CDK (cyclin-dependent kinases). Macrophage and VSMC proliferation seen in Kip1 KO, but also thicker fibrous caps. Intermediate increase for Kip1−/− apoE−/− (424). Somewhat lesser increases in lesions but more inflammation seen in Kip1−/− BMT to apoE−/− (425). Kip1 requires Ser10 phosphorylation for normal suppression of cell proliferation. Ser10Ala mutation leads to increased atherosclerosis with increased ROCK activity (571).</td>
</tr>
<tr>
<td>CDKN2A</td>
<td>CDKN2A−/− apoE−/−</td>
<td>70%</td>
<td>CDKN2A (cyclin-dependent kinase inhibitor 2A) encodes INK4a (also known as p16INK4a) and ARF (also known as p19ARF) through alternative reading frames. Less macrophage apoptosis seen (636). Bone-marrow deficient in INK4a did not affect atherosclerosis (1982), suggesting ARF had the greater effect, consistent with another study showing reduced VSMC and macrophage apoptosis and increased atherosclerosis with ARF deficiency (636).</td>
</tr>
<tr>
<td>CDKN2A</td>
<td>CDKN2A−/− BMT into LDLR−/−</td>
<td>26%</td>
<td>The modest increase in atherosclerosis was accompanied by increase in classically activated M1 macrophages (Ly6C+ and greater tissue accumulation presumably due to reduced INK4a and especially ARF expression. Reverse BMT showed that arterial wall expression of INK4a and ARF did not affect atherosclerosis (3862). Another study found decreased lesion size with CDKN2A KO but no effect of heterozygosity in apoE Leiden mice. CDKN2B and MTAP expression were markedly upregulated in CDKN2A KO mice (908). No effect was seen for ARF KO in that study.</td>
</tr>
<tr>
<td>CDKN2B</td>
<td>CDKN2B−/− in apoE3 transgenic mice</td>
<td>NS</td>
<td>Encodes INK4b. Variable compensatory effects in CDKN2A genes and MTAP were seen making interpretation difficult (908).</td>
</tr>
<tr>
<td>MTAP</td>
<td>MTAP−/− in apoE3 transgenic mice</td>
<td>163%</td>
<td>MTAP (methylthioadenosine phosphorylase) loss or inactivity is associated with several tumors. MTAP is located near the 9p21.3 locus. (908)</td>
</tr>
<tr>
<td>PDGF-B</td>
<td>PDGF-B−/− fetal liver cells transplant into irradiated apoE−/−</td>
<td>NS</td>
<td>Only a temporary delay in VSMC accumulation and fibrous cap formation was seen. Macrophages more inflamed. Similar results seen with blockade of PDGFR signaling (945).</td>
</tr>
<tr>
<td>LRP1</td>
<td>LRP1−/− Cre-Lox in LDLR−/− mice</td>
<td>1510%</td>
<td>LRP1 (LDL receptor-like protein 1) is a transmembrane co-receptor that appears to target the PDGFR receptor. PDGFRβ, for lysosomal degradation. Increased aneurysm formation also seen. With high-cholesterol, high-fat feeding mice generally died in 2 mo with aortic and mesenteric atherosclerotic obstruction (191).</td>
</tr>
<tr>
<td></td>
<td>macrophage-specific LRP1−/− Cre-Lox in mice with either LDLR−/− or apoE−/−</td>
<td>147%</td>
<td>Plaques had more macrophages and fibrosis (790). Similar results in LDLR KO mice with LRP1−/− BMT (1362). Major effect may be due to loss of LRP1 mediation of efferocytosis when activated by apoE with much larger necrotic cores in LRP1−/− LDLR−/− mice (2016). BMT studies suggest macrophage LRP1 inhibits apoptosis whether or not apoE is present (2017). LRP1 internalization defective knock-in also had a 49% increase in atherosclerosis (643). Hepatocyte-specific LRP1 deficiency was atherogenic independent of effects on plasma lipids (485).</td>
</tr>
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## Table 9.—Continued

<table>
<thead>
<tr>
<th>Gene</th>
<th>Model</th>
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<tbody>
<tr>
<td>RAP</td>
<td>RAP&lt;sup&gt;−/−&lt;/sup&gt; LDLR&lt;sup&gt;−/−&lt;/sup&gt; with chronic ANG II infusion</td>
<td>↓ 32%</td>
<td>RAP (receptor associated protein) is an important chaperone for LRP1 (and other receptors), escorting LRP1 from the ER to the Golgi and simultaneously blocking premature ligand binding, allowing effective delivery of LRP1 to the cell surface. RAP deficiency generally leads to impaired LRP1 expression (1045). The unexplained paradoxical decrease in atherosclerosis cited here was shown not to be mediated by any bone marrow derived cells and occurred despite elevations in LDL, VLDL, and probably chyominated remnants (1910).</td>
</tr>
<tr>
<td>LRP5</td>
<td>LRP5&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑ 350%</td>
<td>Increases in TGRL remnants complicates interpretation. Potential for Wnt signaling in VSMC was not examined (1113).</td>
</tr>
<tr>
<td>LRP6</td>
<td>LRP6 R611C in a human pedigree</td>
<td>↑ 1,000%</td>
<td>Risk estimated from report of Iranian pedigree [see text] (1134). Later study showed the R611C LRP6 variant impaired targeting of PDGFRβ by lysosomal degradation in VSMC (896).</td>
</tr>
<tr>
<td>PKG</td>
<td>VSMC-specific CreLox PKG&lt;sup&gt;−/−&lt;/sup&gt; in apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↓ 31%</td>
<td>PKG (cGMP-dependent protein kinase, also known as PRKG1 or cGKI) can facilitate VSMC proliferation, migration, and PKB signaling in VSMC. About 55% of VSMC cells showed effective conditional elimination of PKG in this study and these selectively remained in the media rather than migrating to the intima (1972).</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TRAIL&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑ 26%</td>
<td>TRAIL (tumour necrosis factor-related apoptosis inducing ligand) can have pro- or anti-apoptotic/proliferative effects depending on concentration and context. Shown are results at 8 wk. By 12 wk the effects was NS. TRAIL can support VSMC proliferation but little difference in VSMC seen in lesions (1930).</td>
</tr>
<tr>
<td>TAGLN</td>
<td>VSMC-specific CreLox TAGLN&lt;sup&gt;−/−&lt;/sup&gt; in apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑ 28%</td>
<td>TAGLN (transgelin, also known as SM22 or SM22α) is an actin binding protein expressed in vascular and other SMC. Increased atherosclerosis here was associated with increased proliferation of VSMC with activation markers (509). VSMC in SM22 KO mice had increased NF-κB activation upon carotid arterial injury with greater adhesion molecule expression possibly related to greater ROS production associated with cytoskeletal disruption (1614).</td>
</tr>
<tr>
<td>DDR1</td>
<td>DDR1&lt;sup&gt;−/−&lt;/sup&gt; LDLR&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↓ 60%</td>
<td>DDR1 (discoid domain receptor 1) is a receptor tyrosine kinase that recognizes triple helical collagen types I, VI, and VIII. In VSMC, type I collagen binding to DDR1 inhibits collagen and elastin synthesis as well as VSMC proliferation. In monocytes, differentiation into macrophages is promoted together with production of inflammatory cytokines. Plaques in DDR1&lt;sup&gt;−/−&lt;/sup&gt; mice were smaller and had fewer macrophages through 24 wk (as shown) and had more collagen and elastin at 12 wk (538).</td>
</tr>
<tr>
<td>CLU</td>
<td>CLU&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↓ 52%</td>
<td>CLU (clusterin) is also known as apo J and is found on HDL. CLU-deficient mice showed less VSMC proliferation with a reduction in Egr-1 and TNF-α (701).</td>
</tr>
</tbody>
</table>

### Macrophage inflammation related to lipid accumulation, perpetuation of inflammation

| STAT1       | STAT1<sup>−/−</sup> BMT into apoE<sup>−/−</sup> | ↓ 45% | STAT1 induced transcription for CD36 but not by the SR-A scavenger receptor (15). Plaque apoptosis and necrosis was greatly decreased (1047). |
| NLRP3       | NLRP3<sup>−/−</sup> BMT to LDLR<sup>−/−</sup> | ↓ 69% | Activation of the NLRP3 inflammasome by cholesterol crystals leads to formation of oligomers of NLRP3, ASC, and caspase 1 which can then cleave pro-IL-1β (or pro-IL-1β) to active cytokines. NLRP3<sup>−/−</sup> BMT animals have lower plasma IL-1β as well (452). No effect in apoE KO mice or for caspase 1 deficiency (1193). |
| ASC         | ASC<sup>−/−</sup> BMT to LDLR<sup>−/−</sup>  | ↓ 70% | ASC (apoptosis-associated Speck-like protein containing a CARD [caspase-associated recruitment domain]) is an essential adapter protein in the NLRP3 inflammasome linking NLRP3 to pro-CASP1. Virtually identical reduction in atherosclerosis seen in IL-1α/β KO BMT (452). No effect in apoE KO mice (1193). |
| CASP1       | CASP1<sup>−/−</sup> apoE<sup>−/−</sup>       | ↓ 40% | CASP1 (caspase 1) forms part of the NLRP3 inflammasome as noted above. Reduction in atherosclerosis seen in ascending aorta, not aortic arch and was accompanied by a reduction in IL-1β, IL-1β, IFN-γ, and MHC-II-bearing cells (575). |

Continued
### Table 9.—Continued

<table>
<thead>
<tr>
<th>Gene</th>
<th>Model Description</th>
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<tbody>
<tr>
<td>AIF-1</td>
<td>VSMC-specific Tg in C57BL/6 fed Paigen diet</td>
<td>↑ 151%</td>
<td>AIF-1 (allograft inflammatory factor 1) is a calcium- and actin-binding, cytoplasmic scaffold protein that may promote complex formation with multiple signaling proteins including G proteins such as Rac1 (1652). Increased expression of AIF-1 also leads to greater phosphorylation of Akt, Erk1/2, and p38. Endothelial AIF-1 promotes angiogenesis. Both macrophage and VSMC expression of AIF-1 is increased in human plaques and by incubation with oxLDL. AIF-1 promotes proliferation, migration, and phagocytic activity in these cells. Whole body AIF-1 transgenic, apoE-null mice had a 20% increase in atherosclerosis (1216).</td>
</tr>
<tr>
<td>iNOS</td>
<td>iNOS−/− BMT in apoE−/−</td>
<td>↓ 47%</td>
<td>Also known as NOS2. Shown are results in males. Proatherosclerotic effects of iNOS appear to be primarily related to leukocyte expression. iNOS was shown to produce both NO and superoxide in the lesions (1430). iNOS deletion in normolipidemic mice appears to cause hyperlipidemia and increase atherosclerosis (818).</td>
</tr>
<tr>
<td>S100A9</td>
<td>S100A9−/− apoE−/−</td>
<td>↓ 25%</td>
<td>S100A9 (also known as myeloid-related protein 14) stabilizes S100A8; S100A9 KO leads to undetectable S100A8 as well. S100A8/9/12 are calgranulins which can act as TLR4 and RAGE agonists. S100A9 KO macrophages had reduced cytokine production in response to LPS (356) but VSMC (or endothelial) production may be relevant as well (see S100A12 below). No effect seen with S100A9−/− BMT into LDLR−/− (88).</td>
</tr>
<tr>
<td>S100A12</td>
<td>S100A12 vascular VSMC-specific Tg in apoE−/−</td>
<td>↑ 40%</td>
<td>VSMC-specific overexpression of human S100A12. Plaques showed greatly increased calcification, ROS, and NOD1 activity (773).</td>
</tr>
<tr>
<td>Leptin</td>
<td>ob/ob apoE−/−</td>
<td>↓ 44%</td>
<td>Lep (leptin) deficiency decreased atherosclerosis only after 12 wk with possible early increase of fatty streaks at 4 wk. The ob/ob apoE−/− mice had less fibrous lesions with fewer VSMC at 16 wk. Exogenous leptin increased atherosclerosis in apoE−/− mice (306). Mechanism was unclear: Increased hyperlipidemia and atherosclerosis was seen in ob/ob LDLR−/− mice (730).</td>
</tr>
</tbody>
</table>

#### Macrophage ER stress and apoptosis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Model Description</th>
<th>%Δ A</th>
<th>Function, Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>FABP4</td>
<td>FABP4−/− BMT into apoE−/−</td>
<td>↓ 48%</td>
<td>FABP4 (fatty acid binding protein 4) is found in macrophages as well as adipocytes. Also known as aP2 (adipocyte protein 2). Reduction in atherosclerosis with FABP4 deficient macrophages (1122) was later shown to be likely due to reduced ER stress (484).</td>
</tr>
<tr>
<td>FABP5</td>
<td>FABP5−/− BMT into apoE−/−</td>
<td>↓ 36%</td>
<td>FABP5 (fatty acid binding protein 5) is also known as M1a1 and expressed at about equal molar ratios as FABP4 in macrophages. Reduced macrophage activation seen with decreased PPARγ signaling (95). Combined FABP4/5 deficiency decreased atherosclerosis similarly to FABP4 deficiency but with greater improvement in insulin resistance and increased longevity (178).</td>
</tr>
<tr>
<td>CHOP</td>
<td>CHOP−/− apoE−/−</td>
<td>↓ 35%</td>
<td>CHOP (C/EBP homologous protein) is a key mediator of apoptosis in prolonged ER stress. Plaque necrosis decreased 50% and apoptosis 35%. Similar results in LDLR KO (1789). In BMT experiments, presence of CHOP in macrophages led to much greater caspase 3 activation (a marker of apoptosis) and 4 times more plaque rupture. LDL-derived unesterified cholesterol can trigger ER stress (1819). Other studies suggested vascular wall and particularly endothelial CHOP expression may contribute importantly to atherosclerosis with CHOP-deficient endothelial cells expressing less MCP-1 and VCAM-1 (587).</td>
</tr>
<tr>
<td>XBP1</td>
<td>Spliced (active) XBP1 Tg aorta transplanted into apoE−/−</td>
<td>↑ 837%</td>
<td>Inactive, unprocessed XBP1 (X-box binding protein 1) mRNA is spliced by IRE1 during ER stress. Spliced XBP1 mRNA is translated to an active transcription factor with multiple effects depending on degree and duration of the signal (2060). ER stress found in areas of disturbed flow (329, 1213, 1729).</td>
</tr>
<tr>
<td>Bax</td>
<td>Bax−/− BMT in LDLR−/−</td>
<td>↑ 49%</td>
<td>Bax (Bcl-2-associated X protein) activation promotes apoptosis. Bax KO mice had much less macrophage apoptosis in early lesions which were therefore larger (1060).</td>
</tr>
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### Table 9.—Continued

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<thead>
<tr>
<th>Gene</th>
<th>Model</th>
<th>%Δ A</th>
<th>Function, Comment</th>
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<tbody>
<tr>
<td>Bcl-2</td>
<td>macrophage-specific Bcl-2 deletion [Cre-Lox] in apoE−/− mice</td>
<td>NS</td>
<td>Bcl-2 (B-cell leukemia/lymphoma 2) inhibits apoptosis. More macrophage apoptosis was seen in lesions generally. Increased necrosis and cholesterol crystal formation seen in larger lesions (1790). Overexpression of Bcl-2 in macrophages led to increased lesion size early in atherogenesis but smaller lesions at a later stage (536).</td>
</tr>
<tr>
<td>Aim</td>
<td>Aim−/− LDLR−/−</td>
<td>↓ 73%</td>
<td>Aim (apoptosis inhibitor expressed by macrophages, also known as CDS1) is induced by oxLDL through LXR/RXR. Deletion of Aim promoted early macrophage apoptosis in lesions (74).</td>
</tr>
<tr>
<td>Hmgb1</td>
<td>Hmgb1 neutralizing antibody to apoE−/−</td>
<td>↓ 55%</td>
<td>Hmgb1 (high-mobility group box protein 1) is a DNA binding protein which, when released from necrotic cells (for secreted from various activated cells) acts as a pro-inflammatory, chemoattractant &quot;danger signal&quot; to surrounding cells or phagocytes, possibly acting through RAGE, TLR2, and TLR4 (878).</td>
</tr>
<tr>
<td>Fas</td>
<td>Fas−/− apoE−/−</td>
<td>↑ 70%</td>
<td>These mice (lpr strain) show a lupus-like autoimmunity. Greater apoptosis was seen in lesions which correlated with serum IgG (517). Both increased apoptosis and impaired clearance of apoptotic bodies seen. Similar results were seen in LDLR KO mice transplanted with bone marrow from lupus-susceptible mice (1676).</td>
</tr>
<tr>
<td>Fasl</td>
<td>Fasl−/− apoE−/−</td>
<td>↑ 207%</td>
<td>Another lupus model (gld strain). threefold increase in macrophages and T-cells seen together with more apoptotic cells and reduced effector cell (73).</td>
</tr>
<tr>
<td>Mertk</td>
<td>Mertk−/− BMT into LDLR−/−</td>
<td>↑ 66%</td>
<td>Mertk (Mer receptor tyrosine kinase) interacts with Gas6 to promote effector cell (473). Increased apoptosis and inflammation in lesions in KO seen (32). Kinase defective (KD) Mertk in apo E deficient mice showed more nonmacrophage-associated apoptotic cells, consistent with defective effector cell (1788).</td>
</tr>
<tr>
<td>Gas6</td>
<td>Gas6−/− apoE−/−</td>
<td>NS</td>
<td>Gas6 (growth arrest specific gene 6) recognizes phosphatidylserine on damaged cells and bridges with Mertk. Mice with Gas6 deficiency surprisingly had more fibrous, less inflamed plaques with increased TGFβ production (1095). These findings may be due to role of Gas6 to (through receptors Mertk, Tyro3, and Axl) promote outside-in signaling in platelets (65).</td>
</tr>
<tr>
<td>Mfge8</td>
<td>Mfge8−/− BMT into LDLR−/−</td>
<td>↑ 71%</td>
<td>Mfge8 (milk fat globule-EGF factor 8, also known as lactadherin) bridges phosphatidylserine on apoptotic cells with the αvβ3/5 integrin on phagocytes, promoting effector cell. Results at 8 wk shown. Greatly increased apoptotic debris and markers of apoptosis as well as formation of necrotic core seen in Mfge8 KO mice (31).</td>
</tr>
<tr>
<td>Tg2</td>
<td>Tg2−/− BMT into LDLR−/−</td>
<td>↑ 22%</td>
<td>Tg2 (transglutaminase 2) also cross-links with integrin αvβ3 and promotes effector cell. More plaques with large necrotic cores seen (168).</td>
</tr>
<tr>
<td>Apoe</td>
<td>ApoE−/− BMT into C57BL/6 fed butter fat diet + cholate</td>
<td>↑ 892%</td>
<td>There was no difference in serum lipids in this model. Mice transplanted with WT cells had very little atherosclerosis (502). A later study showed greatly impaired effector cell in ApoE deficient macrophages (552). Apo E also acts with ABCA1 and ABCG1 receptors to promote cholesterol efflux and limit monocyte and neutrophil precursor proliferation (1258). Apo E signaling through macrophage VLDLR or ApoER2 also promotes macrophage conversion from M1 to the less inflammatory M2 phenotype (99).</td>
</tr>
<tr>
<td>Tlr3</td>
<td>Tlr3−/− apoE−/−</td>
<td>↑ 40%</td>
<td>May possibly relate to anti-inflammatory effects of effector cell. Effect seen at 15 wk but became NS by 30 wk. TLR3 (Toll-like receptor 3) detects double-stranded RNA (from viral replication) but dsRNA did not mediate effect in this study. TLR3 ligation may increase IL-10 secretion and expression of PDL1 and PDL2 (coinhibitory receptors) (339). Mild effect recently confirmed in LDLR KO mice (1484).</td>
</tr>
</tbody>
</table>
more physiological model of adenoviral delivery of ApoE into ApoE\(^{-/-}\) mice to rapidly normalize lipid levels. CE content of plaques dropped within 2 wk followed by a marked reduction in macrophage recruitment rates into the plaques and greatly reduced macrophage content by 4 wk. Macrophage egress did not change over this period and was quantitatively minimal while apoptosis rate continued steadily in both the newly normolipidemic mice versus their counterparts with continuing hyperlipidemia. Furthermore, the reduction in macrophage content did not depend on CCR7 as in the previous transplant study. It was thought that adventitial inflammation from transplantation of the vessel may have somehow accelerated macrophage egress in that model, a condition thought to be much less relevant to the usual human situation. Thus, rather than coaxing faster macrophage egress from plaques, lipid normalization leads to marked reduction in macrophage recruitment as the main means to effect plaque regression. These findings also suggest that slowing monocyte/macrophage entry into lesions would be an important means to facilitate regression.

As alluded to earlier in this review, apoptosis of macrophages early in plaque development seemed to minimize...
### Table 10. Plaque destabilization, thrombosis, and precipitation of acute events (46 genes tested)

<table>
<thead>
<tr>
<th>Gene</th>
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<tbody>
<tr>
<td>MMP1</td>
<td>macrophage-specific MMP1 transgenic in apoE−/−</td>
<td>↓32%</td>
<td>Also known as collagenase. Mice do not possess a MMP1 homolog. Over-expression of MMP1 decreased plaque size and collagen though without rupture (1001).</td>
</tr>
<tr>
<td>MMP2</td>
<td>MMP2−/− apoE−/−</td>
<td>↓49%</td>
<td>Smaller plaque size seen but much greater reduction in VSMC than macrophages with thinner fibrous cap (966)</td>
</tr>
<tr>
<td>MMP3</td>
<td>MMP3−/− apoE−/−</td>
<td>↑343%</td>
<td>Also known stromelysin-1. MMP3 activates other MMPs. Lesions from MMP3 KO mice were not only larger but had fewer VSMC and showed greater instability (860). Consistent with prior findings of larger plaques but the MMP3 KO mice had fewer aortic aneurysms (1633). MMP3 also appears necessary for outward remodeling (47).</td>
</tr>
<tr>
<td>MMP7</td>
<td>MMP7−/− apoE−/−</td>
<td>↓7%</td>
<td>No significant change in lesion size or stability seen despite 78% increase in VSMC (860).</td>
</tr>
<tr>
<td>MMP9</td>
<td>MMP9−/− apoE−/−</td>
<td>↑122%</td>
<td>Also known as gelatinase B. KO resulted in increased plaque size, fewer VSMC, more macrophages, and apparent decreased plaque stability (860). Overexpression of a self-activating form of MMP9 did result in plaque disruption, however (648). In moderate to advanced lesions, adenoviral expression in plaques led to plaque neovascularization and intraplaque hemorrhage (396).</td>
</tr>
<tr>
<td>MMP12</td>
<td>MMP12−/− apoE−/−</td>
<td>↓52%</td>
<td>In MMP12 KO mice, plaques had 2.5-fold more VSMC and a 53% decrease in macrophage content as well as decreased overall size. Plaques were also more stable (860). Other studies suggest MMP12 is critical for macrophage migration. Selective inhibition decreased plaque as well (859).</td>
</tr>
<tr>
<td>MMP13</td>
<td>MMP13−/− apoE−/−</td>
<td>NS</td>
<td>No difference in size or cellular composition but increased plaque collagen (402).</td>
</tr>
<tr>
<td>MMP14</td>
<td>MMP14−/− BMT into LDLR−/−</td>
<td>NS</td>
<td>Similar phenotype to MMP13 KO with increased plaque collagen (1560).</td>
</tr>
<tr>
<td>TIMP1</td>
<td>TIMP1−/− apoE−/−</td>
<td>NS</td>
<td>TIMP1 is on the X chromosome. Female TIMP1 KO were generated then male offspring lacking TIMP1 were used for studies. TIMP1 KO had no effect on lesion size but gelatinase activity was greater with increased medial lamina ruptures (1002). Others showed similar results (858, 1632).</td>
</tr>
<tr>
<td>TIMP2</td>
<td>TIMP2 adenoviral gene transfer at 7 wk in apoE−/−</td>
<td>↓60%</td>
<td>Apparent arrest of atherosclerosis progression seen. Marked decrease in apoptosis, macrophages, and buried fibrous caps, with increased VSMC and fibrous tissue (858).</td>
</tr>
<tr>
<td>TIMP3</td>
<td>macrophage-specific TIMP3 Tg in LDLR−/−</td>
<td>↓48%</td>
<td>Macrophage-specific overexpression of TIMP3 reduced atherosclerosis at 16 and 24 wk. Necrotic core was greatly reduced and collagen content was increased. TIMP3 inhibits ADAM17 as well as MMP2/9/14 (262).</td>
</tr>
<tr>
<td>CatK</td>
<td>CatK−/− apoE−/−</td>
<td>↓42%</td>
<td>Cathepsin K (1094).</td>
</tr>
<tr>
<td>CatL</td>
<td>CatL−/− LDLR−/−</td>
<td>↓77%</td>
<td>Cathepsin L. Required for angiogenesis and presumably vaso vasmor formation. Degradates extracellular matrix elastin and collagen. Deficiency results in impaired VSMC, macrophage, and T-cell migration (917).</td>
</tr>
<tr>
<td>CatS (CTSS)</td>
<td>CatS−/− LDLR−/−</td>
<td>↓30%</td>
<td>Cathepsin S3 is one of the most potent elastases known. Deficiency reduced macrophage, VSMC, lipid, and collagen content of plaques. In vitro migration of macrophages impaired (1701). May also prime LDL for further modification by sPLA2-V and soluble sphingomyelinase (1421).</td>
</tr>
<tr>
<td>Cst3</td>
<td>Cst3−/− apoE−/−</td>
<td>NS</td>
<td>Cst3 (Cystatin C), a cysteine protease inhibitor, is an endogenous inhibitor of cathepsins. Cyst C KO did not affect lesion size but led to plaques rich in collagen. However, there was evidence for greater elastin degradation and progressive aortic dilation (1700).</td>
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Table 10.—Continued

<table>
<thead>
<tr>
<th>Gene</th>
<th>Model Description</th>
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<tbody>
<tr>
<td>vWF</td>
<td>vWF&lt;sup&gt;−/−&lt;/sup&gt; LDLR&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↓ 40%</td>
<td>Lesions in vWF&lt;sup&gt;−/−&lt;/sup&gt; were particularly less frequent at the usual branch point locations. ApoE KO vWF&lt;sup&gt;−/−&lt;/sup&gt; mice showed a similar effect. Early reduction in atherosclerosis but the protection did not persist at later time points (1200). vWF could also be considered an initiating factor since its adherence to athero-prone endothelial sites can promote platelet adherence with subsequent endothelial activation and leukocyte adherence (806)[Gandhi, 2012 #48589.</td>
</tr>
<tr>
<td>ADAMTS13</td>
<td>ADAMTS13&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑ 60%</td>
<td>ADAMTS13 is made in liver and by endothelial cells. Its only known substrate are ultra large multimers of vWF. This form of vWF is released from Weibel-Palade bodies and promotes platelet adhesion which can, in turn promote monocyte and other leukocyte adhesion (584).</td>
</tr>
<tr>
<td>TF</td>
<td>TF&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>NS</td>
<td>Complete TF (tissue factor) deficiency is embryo lethal. There were no differences in extent or composition of plaques in either model (1794). A SMC Cre-Lox KO model showed reduced injury-induced thrombosis, but possible effects on atherosclerosis have not yet been reported (1902).</td>
</tr>
<tr>
<td>TFPI</td>
<td>TFPI&lt;sup&gt;+/+&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑ 29%</td>
<td>TFPI&lt;sup&gt;−/−&lt;/sup&gt; is embryonic lethal (as is tissue factor KO). TFPI&lt;sup&gt;+/+&lt;/sup&gt; mice showed slower thrombus formation after photochemical injury to plaques. Only carotid and iliac atherosclerosis was significantly more extensive (shown is aortic atherosclerosis) (1494).</td>
</tr>
<tr>
<td>Factor II</td>
<td>F2&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↓ 44%</td>
<td>Homozygous FII (prothrombin) deficiency is embryonic lethal. About 50% reduction in plasma prothrombin seen with more stable plaque in F2&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt; mice (182).</td>
</tr>
<tr>
<td>Factor V</td>
<td>F5 Leiden (equivalent) apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑ 57%</td>
<td>Plaques were larger but had smaller necrotic cores, thicker fibrous caps, more VSMC, fewer macrophages, and fewer ruptures (1581).</td>
</tr>
<tr>
<td>Factor VIII</td>
<td>F8&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↓ 40%</td>
<td>Much less fibrin in lesions (889).</td>
</tr>
<tr>
<td>PAR1</td>
<td>PAR1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>—</td>
<td>Tendency for increased neointimal formation after carotid injury (305). PAR1 (protease-activated receptor 1) is not expressed on mouse platelets as it is in humans (1120). It is also a target of activated protein C.</td>
</tr>
<tr>
<td>PAR2</td>
<td>PAR2&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>—</td>
<td>Decreased neointimal thickness after arterial injury (1768) PAR2 is not expressed on endothelial cells and various leukocytes. May be activated by factors VIIa and Xa while thrombin activates all other PARs [1, 3, 4] (1120). Activation by trypsin caused endothelial activation, ROS generation, and lymphocyte adhesion (1046, 1120).</td>
</tr>
<tr>
<td>PAR3</td>
<td>PAR3&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>—</td>
<td>Mice protected against thrombosis. Atherosclerosis not tested (1939).</td>
</tr>
<tr>
<td>PAR4</td>
<td>PAR4&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>NS</td>
<td>No effect at any site despite protection from thrombosis (703).</td>
</tr>
<tr>
<td>TM</td>
<td>TM&lt;sup&gt;Pro/Pro&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑ 67%</td>
<td>TM (thrombomodulin) protein mutation impairs activation of protein C and increases thrombin activity. TM mutant mice had larger nonobstructive plaques but they were more stable (1581). No effect on atherosclerosis with human TM knock-in with defective protein C activation (1451).</td>
</tr>
<tr>
<td>Protein C</td>
<td>PC&lt;sup&gt;−/+&lt;/sup&gt;</td>
<td>—</td>
<td>Increased inflammation of intimal proliferation with copper/silicone arterial cuff model (265).</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>Fbg&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>NS</td>
<td>No difference at 31 wk. Slightly smaller lesions in Fbg&lt;sup&gt;−/−&lt;/sup&gt; mice were seen at 22 wk (1992). Fbg overexpression had no effect in apo E3-Leiden mice (1481). An 81% reduction in lesion size was seen for fibrinogen deficiency in human Lp(a) transgenic mice (1078) while a 15% increase in aortic lesion size were seen in LDLR&lt;sup&gt;−/−&lt;/sup&gt;/ ApoBEC1&lt;sup&gt;−/−&lt;/sup&gt; mice (835).</td>
</tr>
<tr>
<td>Plasminogen</td>
<td>Plg&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑ 620%</td>
<td>Plasminogen may be protective in artery wall since fibrinopeptides generated by plasmin may actively signal to VSMC to generate anti-inflammatory TGFβ (1993). In another model, with scavenger receptor promoter-driven macrophage over-expression of uPA, plasminogen appeared pro-atherogenic and plasminogen KO decreased atherosclerosis, though animals with uPA over-expression had much more atherosclerosis (948).</td>
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### Table 10.—Continued

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<thead>
<tr>
<th>Gene</th>
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<tr>
<td>tPA (PLAT)</td>
<td>tPA&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;3&lt;/sup&gt;-Leiden</td>
<td>↓ 70%</td>
<td>Advanced lesions were particularly decreased while there was an increase in very early lesions in tPA (tissue-type plasminogen activator) KO. Less collagen seen. tPA may be a VSMC mitogen (1480). Slight increase in injury-induced carotid stenosis seen in tPA-deficient (1549).</td>
</tr>
<tr>
<td>uPA (PLAU)</td>
<td>uPA&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;3&lt;/sup&gt;-Leiden</td>
<td>NS</td>
<td>Whole body uPA (urokinase-type plasminogen activator) deficiency had no effect on atherosclerosis in the aortic root (408, 1480) but increased plaque size in some other areas and decreased plaque stability (408). Macrophage-specific over-expression in apoE&lt;sup&gt;−/−&lt;/sup&gt; mice increased plaque (142%) and greatly increased coronary lumen narrowing with 62% stenosis in transgenics versus just 2.6% in controls—with many myocardial infarctions and early mortality (354). Later studies showed uPA over-expression effect was dependent on plasminogen and could be evoked by BMT into older apoE&lt;sup&gt;−/−&lt;/sup&gt; mice (789, 948). BMT with uPA KO led to less atherosclerosis only in older mice with established lesions (953). Generated plasmin can activate MMPs leading to fibrous cap rupture and intraplaque hemorrhage (789).</td>
</tr>
<tr>
<td>α&lt;sub&gt;2&lt;/sub&gt;-AP</td>
<td>α&lt;sub&gt;2&lt;/sub&gt;-AP&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>—</td>
<td>α&lt;sub&gt;2&lt;/sub&gt;-AP (α&lt;sub&gt;2&lt;/sub&gt;-antiplasmin) is the main physiological direct plasmin inhibitor. No effect on neointimal formation after electrical injury to the femoral artery was seen (1044).</td>
</tr>
<tr>
<td>HCF2</td>
<td>HCF2&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑ 54%</td>
<td>HCF2 (heparin cofactor 2) is an endogenous thrombin inhibitor. It is also known as serpin D1. The ability of HCII to block thrombin is greatly increased by dermatan sulfate or heparin. Besides larger plaques, animals showed greater VSMC proliferation after mechanical artery injury (1876). Similar results shown with heterozygous deficiency (23).</td>
</tr>
<tr>
<td>FBN1</td>
<td>FBN1 C1039G&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑ 179%</td>
<td>FBN1 (fibrillin-1) haploinsufficiency due to this mutation can cause Marfan’s (865). Cross with apoE KO leads to increased plaque size and instability with frequent plaque rupture in advanced lesions with cerebral infarction (1857).</td>
</tr>
<tr>
<td>HPS1</td>
<td>pale ear mice fed Paigen diet</td>
<td>↑ 72%</td>
<td>HPS1 (Hermanak-Pudlik syndrome 1, the gene corresponding to pale ear mouse) is a transmembrane protein in the BLOC-3 complex required for normal biogenesis of lysosome-related organelles, including melanosomes and platelet dense granules (801). HPS1 patients have a bleeding diathesis as well as ocular-cutaneous albinism and ceroid storage disease (which can result in pulmonary fibrosis and granulomatous colitis). Mice were congenic with C57BL/6 which served as control. Despite abnormal platelet function, atherosclerotic lesions were larger though area involved was not different in this strain (1370).</td>
</tr>
<tr>
<td>HPS3</td>
<td>HPS3&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt; after FeCl&lt;sub&gt;3&lt;/sub&gt;&lt;sup&gt;−/−&lt;/sup&gt; induced arterial injury</td>
<td>↓ 65%</td>
<td>HPS3 (cocoa mouse) has platelet dysfunction but is otherwise milder than other forms of HPS. Given here is the reduction in the intima/media ratio (remodeling) 21 days after carotid injury induced by surrounding the artery for 3 min with filter paper soaked in 5% FeCl&lt;sub&gt;3&lt;/sub&gt;. The HPS3&lt;sup&gt;−/−&lt;/sup&gt; mice also had greatly reduced acute thrombus formation immediately after the injury (912).</td>
</tr>
<tr>
<td>HPS4</td>
<td>light ear mice fed Paigen diet</td>
<td>↓ 53%</td>
<td>HPS4 (light ear mouse) had reduced lesion area and size (1370).</td>
</tr>
<tr>
<td>HPS5</td>
<td>maroon mice fed Paigen diet</td>
<td>↓ 45%</td>
<td>HPS5 (ruby-eye 2 or maroon mouse) had reduced lesion area and size (1370).</td>
</tr>
<tr>
<td>HPS6</td>
<td>ruby-eye mice fed Paigen diet</td>
<td>↓ 59%</td>
<td>HPS6 (ruby-eye mouse) had reduced lesion area and size (1370).</td>
</tr>
</tbody>
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**Vaso-vasorum and angiogenesis related**

<table>
<thead>
<tr>
<th>Gene</th>
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<tbody>
<tr>
<td>VEGF</td>
<td>Adenoviral gene transfer of VEGF-A, -B, -C, or -D with transient strong plasma elevations had no effect on lesions (1008). A prior study had shown marked increase in lesion area after a single injection of human recombinant VEGF into apoE&lt;sup&gt;−/−&lt;/sup&gt; mice and rabbits (266).</td>
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### Table 10.—Continued

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<tr>
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<th>Model</th>
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<tbody>
<tr>
<td>PIGF</td>
<td>PIGF&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↓ 41%</td>
<td>Placental growth factor (PIGF) is a VEGF homologue and acts through its receptor VEGFR1. It promotes angiogenesis but also causes macrophage proliferation. Given are results at 10 wk. By 25 wk a nonsignificant 13% reduction in mean plaque area was seen, suggesting VEGFR1 signaling may only be important in early stages of atherosclerosis. Adenoviral over-expression increased lesions (903). Expression is increased by several inflammatory signals including ANGII, possibly through NF-κB signaling (1374).</td>
</tr>
<tr>
<td>VEGFR1</td>
<td>anti-VEGFR1 antibody in apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↓ 50%</td>
<td>VEGFR1 is also known as Flt1 (flk-like tyrosine kinase 1) is a receptor tyrosine kinase that appears to mediate the pro-atherogenic, angiogenic signaling of PIGF but also binds VEGF-A. Anti-VEGFR1 (1097) treatment decreased atherosclerosis in apo E null mice. The extracellular domain can be generated separately by alternative splicing and is found in plasma as a soluble factor (sFlt-1), sFlt-1 acts as an endogenous inhibitor of PIGF. Reduced levels of sFlt-1 were found in renal disease and were associated with more human CAD. Infusion of sFlt-1 in 5/6 nephrectomized apo E null mice decreased atherosclerosis (1347).</td>
</tr>
<tr>
<td>G-CSF</td>
<td>G-CSF sq injections into apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑ 100%</td>
<td>Mice treated for 8 wk on high fat diet (691). Increased adventitial vascularity. Also 153% increase with GM-CSF injections in same study. See Table 7 for GM-CSF.</td>
</tr>
<tr>
<td>nAChR&lt;sub&gt;a1&lt;/sub&gt;</td>
<td>Aortic RNAi knockdown of nAChR&lt;sub&gt;a1&lt;/sub&gt; in apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↓ 42%</td>
<td>nAChR&lt;sub&gt;a1&lt;/sub&gt; (nicotinic acetylcholine receptor α1) mediates angiogenesis induced by nicotine (which also promotes plaque progression) and urokinase (2069). Endothelial cells store acetylcholine and could be an autocrine source of activation (992).</td>
</tr>
<tr>
<td>PAI-1</td>
<td>PAI-1&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt; or LDLR&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑ 13%</td>
<td>PAI-1 (plasminogen activator inhibitor 1) KO had a nonsignificant effect (1637). No effect of PAI-1 KO in apo E3-Leiden mice (1480). Another group reported significantly increased progression of advanced plaque in PAI-1 KO (1096).</td>
</tr>
<tr>
<td>rPAI-1&lt;sub&gt;23&lt;/sub&gt;</td>
<td>Intraperitoneal injections in LDLR&lt;sup&gt;−/−&lt;/sup&gt; and ApoB-48- deficient mice</td>
<td>↓ 73%</td>
<td>rPAI-1&lt;sub&gt;23&lt;/sub&gt; is a cleavage product of PAI-1 shown to be a major stimulus of plaque angiogenesis. Intraperitoneal injections begun at 14 wk and atherosclerosis measured at 14 and 20 wk. Shown is reduction compared to control mice at 20 wk. Lesions in treated mice at 20 wk were actually smaller than mice at 14 wk and showed a 49% reduction in cholesterol content compared to control, 20-week mice (446).</td>
</tr>
<tr>
<td>Hp</td>
<td>human Hp 2-2 Tg in apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>NS</td>
<td>Hp (haptoglobin) binds hemoglobin and facilitates uptake of the complex via the CD163 scavenger receptor. Related to phagocytosis of Hgb from extravasated RBC. Only humans express the Hp 2 allele which has been associated with much lower stimulation of IL-6 and IL-10 by phagocytosing macrophages in vitro as compared to Hp 1 (668). While there was no difference in atherosclerosis extent, plaques in mice engineered to express Hp 2-2 had more macrophages, iron, and lipid peroxidation products, suggesting a greater potential for instability (1013).</td>
</tr>
<tr>
<td>HAMP</td>
<td>adenoviral HAMP Tg in carotid artery of apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>NS</td>
<td>Extracellular HAMP (hepcidin antimicrobial peptide) binds the plasma membrane iron transport ferroportin and promotes its internalization and degradation causing retention of intracellular iron. While plaques were not larger, they showed increased inflammatory markers and vulnerability. (1025)</td>
</tr>
<tr>
<td>Col18a1</td>
<td>Col18a1&lt;sup&gt;−/−&lt;/sup&gt; apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↑ 67%</td>
<td>Col18a1 encodes collagen XVIII. Increased angiogenesis was seen in the Col18a1 KO mice (1242).</td>
</tr>
<tr>
<td>Endostatin</td>
<td>Endostatin subcutaneous injections into apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↓ 85%</td>
<td>Shown is the decrease in progression of already advanced lesions (started at 20 wk) (1241). Endostatin is a naturally occurring angiogenesis inhibitor derived from the carboxy terminal fragment of collagen XVIII found in vascular extracellular matrix.</td>
</tr>
<tr>
<td>Angiostatin</td>
<td>Angiostatin subcutaneous injections into apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>↓ 31%</td>
<td>Shown are absolute differences 75 days after angiostatin injections were began at 32 wk (1243). Angiostatin is a proteolytic fragment of plasminogen and a natural angiogenesis inhibitor.</td>
</tr>
</tbody>
</table>

References are given in parentheses.
subsequent plaque growth, whereas increased apoptosis in more advanced plaques may saturate removal mechanisms for apoptotic cells and promote growth of lesions and greater inflammation. Direct evidence for this hypothesis comes from a study in ApoE-deficient protein Bcl-2. At 5 wk, lesions were larger and contained more macrophages while by 15 wk, lesions were actually smaller and fewer macrophages were present (596). This principle of early apparent benefit but long-term harm from increased macrophage apoptosis harmonizes a number of apparently contradictory findings (1863).

A newly recognized function of ApoE relates to proliferation and mobilization of monocytes and neutrophils. Several models of hyperlipidemia, including cholesterol and saturated fat-fed pigs, rabbits, and especially ApoE-deficient mice are accompanied by pronounced monocytosis and neutrophilia. Monocytosis was also pronounced in mice with combined deficiency of the ABCA1 and ABCG1 receptors which are involved in export of free cholesterol from various cell types. The monocytosis in these mice was related to cell membrane accumulation of free cholesterol, increased lipid raft formation, and hypersensitivity to the proliferative effects of the GM-CSF (also known as IL-3) receptor (CSF2RB) in hematopoietic stem cells, with CSF2RB ligation leading to ERK and STAT5 activation (1258). Further studies in ApoE KO mice fed a Western-type diet showed that the three- to fourfold increase in blood neutrophils and monocytes was due to impaired cholesterol efflux from hematopoietic stem cells. Quiescence of these cells was normally maintained by cholesterol efflux and controlled plasma membrane cholesterol content through LXR-dependent production of ApoE, ABCA1, and ABCG1. ApoE is normally released from the cell and bound to cell surface heparan proteoglycans where it can immediately interact with ABCA1 and ABCG1 transporters to promote cholesterol efflux. Treatment with heparinase impaired this pathway and promoted proliferation through increased GM-CSF receptor activity leading to intracellular STAT5 and ERK activation. In ApoE-deficient mice, high doses of a recombinant HDL having high cholesterol mobilization capacity (with APOA1 and phospholipid but no cholesterol) could also suppress proliferation. Interestingly, transplant of ApoE-deficient bone marrow cells into LDLR KO mice led to greater hematopoietic cell proliferation, leukocytosis, and proportionately increased deposition of monocytes into atherosclerotic lesions despite similar cholesterol levels (1258). Thus the monocytosis of ApoE-deficient mice may directly contribute to the rapid development of atherosclerosis in these animals. These studies may provide additional insights into previously noted increased risk of CAD associated with higher leukocyte and especially monocyte counts noted in epidemiologic studies (340, 378).

2. Control of proliferation: relationship to atherosclerosis

In advanced lesions, both macrophages and VSMC proliferate. A series of newer studies have demonstrated interesting biological features of pathways controlling cell proliferation, but their direct clinical relevance to human atherosclerosis remains somewhat uncertain. In general, genetic manipulations resulting in greater macrophage and VSMC proliferation typically increase plaque size, but with regard to VSMC, such plaques may be more stable. Also, proliferation is often accompanied by protection from apoptotic signals. In early atherosclerosis, greater apoptosis generally leads to smaller plaques. However, in advanced disease, increased apoptosis can overwhelm efferocytosis pathways, promote secondary necrosis, and lead to increased inflammation with unstable plaques. Pathways related to control of proliferation are reviewed in the following paragraphs.

Cell replication is initiated by extracellular growth factors that signal to ultimately stimulate the transcription of cyclin D together with a reduction of specific cyclin-dependent kinase (CDK) inhibitors (136). In general, CDKs bind cyclins and then phosphorylate other proteins to promote specific steps through the cell cycle. Cyclin D binds CDK4/6 and remains high throughout the cell cycle while discrete waves of other cyclins peak at specific phases of the cycle. Presiding over this system is the transcription factor p53, whose elevation leads to increased production of CDK inhibitors, other proteins that inhibit the cell cycle, DNA repair enzymes, and proapoptotic proteins. In its role, p53 has been dubbed the “guardian of the genome.”

To replicate, the cell must pass through a series of checkpoints that are controlled by a series of serine/threonine kinases known as checkpoint kinases. Ataxia telangiectasia mutated (ATM) is a major checkpoint kinase that is central to a network of proteins that control cell replication, energy production, protein synthesis, autophagy, and apoptosis (126, 1461). The kinase activity of ATM is controlled by ROS and surveillance proteins which bind double-stranded breaks in DNA and recruit and activate ATM. ATM then phosphorylates and activates Chk1/2 (checkpoint kinases 1 and 2). These phosphorylate the transcription factor p53, thereby inhibiting p53 ubiquitination and degradation. ATM also directly phosphorylates and protects p53 and phosphorylates LKB1 (liver kinase B1). Besides AMP or ADP, LKB1 is the main activator of AMPK. Active phosphorylated LKB1 binds to the AMPKγ regulatory subunit, rendering the AMPKα subunit susceptible to other activating phosphorylation (45).

Active ATM and increased p53 generally limit cell growth and mitogenesis while their reduction will promote proliferation. Accordingly, reduction of ATM promoted athero-
sclerosis with increased macrophage proliferation (and JNK activation). In contrast, chloroquin, which activates ATM, reduced atherosclerosis but only in ATM−/− ApoE−/− mice (1561), an effect that also required p53 (1465).

Prior to initiation of G1 (growth phase 1), hypophosphorylated Rb (retinoblastoma gene) sequesters the transcription dimer E2F/DP which is needed for transcription of genes required in the G1 phase of cell replication, thereby blocking growth and proliferation. At the beginning of the cell cycle, CDK4/6:cyclin D and CDK2:cyclin E complexes hyperphosphorylate Rb which then releases E2F/DP, allowing the cell to enter G1 (570). Macrophage Rb KO resulted in increased lesion size with more macrophages (163).

A series of CKI (CDK inhibitor) proteins can bind to and inhibit multiple CDKs throughout the cell cycle and cause cell cycle arrest. Some of the more extensively studied CKIs include Cip1 (also known as p21Cip1), Kip1 (also known as p27Kip1), INK4a (inhibitor of kinase 4a, encoded by the gene CDKN2A, also known as p16INK4a), ARF (alternate reading frame, also known as p19ARF and encoded by CDKN2A), and INK4b (encoded by CDKN2B, also known as p15INK4b). ARF also inhibits MDM2 (mouse double minute 2 homologue) which is an E3 ubiquitin ligase that targets p53 for proteasome destruction. Thus active ARF both directly causes cell cycle arrest as a CKI and promotes accumulation of p53 which also blocks replication and can promote apoptosis.

Single nucleotide polymorphisms (SNPs) within the 9p21.3 locus have been the most consistently identified gene variants associated with CAD in human GWAS (776). Associations are also reported with atherosclerotic stroke, peripheral artery disease, and aneurysms. The 3′ portion of ANRIL (antisense noncoding RNA in the INK4 locus) is the lone occupant of the 9p21.3 atherosclerosis association region. Nearby are the CDKN2A (INK4a, ARF), INK4b, and methylthioadenosine phosphorylase (MTAP) genes. The proximity of 9p21.3 to various other genes that may be involved in atherogenesis makes many animal studies difficult to interpret.

ANRIL is of one several long, noncoding RNA now described. While not encoding a protein, these segments of DNA are transcribed by RNA polymerase II into RNA and then spliced into various isoforms with many of the splicing variants being polyadenylated. The RNA products of ANRIL seem to direct the assembly of two polycomb protein repressive complexes that are involved in methylaing and ubiquitinating histones to repress and maintain repression of targeted genes. The control of splicing and isoform expression and the gene targets are likely cell- and condition-specific. Among the targets of these repressive complexes are the CDKN2A/B loci (349).

The promoter region of ANRIL is rich in binding sites for multiple enhancers. Importantly, several of the disease-associated alleles in the 9p21.3 region are located within these enhancer regions. For example, STAT1 binds to the ANRIL promoter where two human CAD-associated variants occur which impair STAT1 binding (717). In endothelial cells, STAT1 normally increases transcription of ANRIL followed reduced expression of CDKN2B. However, very different effects can be seen in other cell types (349). Furthermore, ANRIL expression can affect the expression of numerous other genes that may be involved in atherogenesis (347). This findings and the fact that mice do not have a locus entirely orthologous to ANRIL make many animal studies difficult to interpret.

Several human studies find reduced expression of ANRIL in association with CAD, carotid atherosclerosis, or CAD-associated 9p21.3 variants, but effects on proliferation of various cells are quite variable (348, 775, 856, 1063). One group reported that the type I interferon IFNo21 was elevated ~10-fold in human carriers of the at-risk alleles (55). Another group reported increased expression of ANRIL exons 1–5 in lymphocytes and greater DNA methylation of INK4b in CAD patients and associations with a major 9p21.3 allele (2115). In primary cultures of human VSMC, at-risk 9p21.3 alleles were associated with decreased ANRIL long transcript expression together with a reduction in INK4a and INK4b expression resulting in greater proliferation. Observations in human coronary plaques were consistent with these in vitro findings, with at-risk alleles being associated with increased VSMC content. It was pointed out that these results were consistent with prior studies suggesting decreased expression of the ANRIL long transcript in association with at-risk 9p21.3 alleles while short ANRIL transcripts were actually increased and suppressed CDKN2A/B products and promoted proliferation (1239). However, a recent report from the Framingham Heart Study reported reduced ANRIL short isoforms in lymphocytes in association with at-risk variants (856). These reports are, as yet, difficult to reconcile.

In mice, transplant of bone marrow cells with heterozygous deficiency of CDKN2A (with its two products INK4a and ARF which inhibit proliferation) was sufficient to modestly increase atherosclerosis (by ~26%). Only bone-marrow derived cells with the CDKN2A deficiency affected atherosclerosis. Mice heterozygous for the defective gene showed an increase in the proportion of classically activated, circulating monocytes (Ly6C+6) as well as increased proliferation of activated macrophages (962). How these and other results in mice (see
TABLE 9) relate to the human 9p21.3 associations with CAD remains to be fully demonstrated.

3. Newer insights into the role of VSMC in atherosclerosis

The notion of platelets releasing PDGF after adhering to areas of denuded endothelium with PDGF then causing vascular VSMC proliferation was a central feature of the original formulation of the “response to injury” hypothesis (1512, 1513). Since that time, the focus on more traditional inflammatory cells has perhaps obscured the role of VSMC in atherosclerosis. Recently, data were put forward to suggest that most proliferating or secretory VSMC may actually be derived from stem cells residing in the arterial media. These stem cells appeared to differentiate into cells with a smooth muscle cell phenotype only after activation and transmigration into the plaque (1755). Others have demonstrated that, in response to proliferation and phenotype switching cues such as PDGF and oxidized phospholipids, VSMC can lose cellular markers to the point that they are virtually indistinguishable from macrophages. Furthermore, macrophages can acquire some markers generally assigned to cells of VSMC origin (633). Continued research will undoubtedly refocus attention on the importance of lesional cells with apparent VSMC phenotype. No distinction regarding the origin of lesional VSMC is made in the following discussion, although this may become an issue of great interest in future research.

VSMC are among the first cells found in the intima in locations destined to become atherosclerotic plaques, presumably in response to PDGF-BB produced initially by activated endothelial cells in regions of disturbed flow. Beyond being a source for extracellular matrix and collagen, in an activated, secretory state VSMC can elaborate chemokines including MCP-1 and CX3CL1 (fractalkine), as well as adhesion molecules (VCAM-1, ICAM-1) which attract and then capture macrophages within the intima. VSMC can synthesize and secrete pro-inflammatory cytokines including IFN-γ, IL-1, IL-6, and IL-18. They can alter the extracellular matrix to further promote inflammation by producing fibronectin. They express various scavenger receptors and take up modified lipoproteins to become foam cells which can cause further activation. Thus VSMC can take on characteristics of endothelial cells in recruitment of leukocytes, some of the functions of T-cells by producing cytokines to amplify the inflammatory response, and the properties of macrophages by conversion to foam cells and elaboration of similar cytokines. More recently, VSMC have also been observed to recapitulate additional functions generally attributed to macrophages, such as efficient phagocytosis of apoptotic cells with secretion of inflammation-resolving (although pro-fibrotic) cytokines including PDGF and TGF-β (438).

VSMC are normally potent, effective phagocytes of apoptotic VSMC. This capacity of VSMC for avid efferocytosis has been previously unappreciated. Studies in mice engineered with inducible, VSMC-specific diptheria toxin receptor provide new insights into an important role for VSMC in advanced plaques as active phagocytes. Low-level diptheria toxin administration to these mice resulted in apoptosis in ~1% of VSMC, a rate similar to human plaques. In normolipidemic mice, this degree of apoptosis resulted in no obvious inflammation. However, in the face of hyperlipidemia, normal phagocytosis of apoptotic VSMC by other VSMC was severely impaired (332). Failed phagocytosis of apoptotic VSMC by surrounding normal VSMC resulted in secondary necrosis and markedly increased production of inflammatory cytokines by necrotic VSMC (including IL-1α, IL-1β), followed by greater IL-6 and MCP-1 production by surviving VSMC (332). A twofold increase in atherosclerosis with impaired outward remodeling and increased stenosis ensued (331).

On the basis of the above results, antiapoptotic signaling in VSMC (and macrophages) might be expected to limit atherogenesis through promotion of efficient efferocytosis. Antiapoptotic signaling by activation of PKB is a primary result of intracellular signaling stimulated by several growth factors. PKB (Akt1) KO caused thinning of fibrous caps, plaque rupture, and spontaneous MI in ApoE-null mice (520). Binding of IGF-I to its receptor IGF-IR (a typical receptor tyrosine kinase) results in activation of PKB in a fashion essentially identical to the insulin receptor. Infusion of IGF-I into ApoE-null mice modestly reduced atherosclerosis, decreased vessel wall ROS (probably by PKB phosphorylation and stimulation of eNOS), and reduced macrophage infiltration into the vessel wall (1699). However, VSMC-specific overexpression of IGF-I did not alter atherosclerosis burden, although it did increase plaque stability (1598). Knockout of macrophage IGF-IR resulted in a substantial increase in atherosclerosis, suggesting a protective effect of IGF signaling in these cells (762).

These results contrast with the consistent increase in neointimal formation after arterial injury that generally accompanied augmented IGF-I signaling, illustrating some of the major differences between atherosclerosis and vascular injury repair (763). However, excessive IGF-IR signaling may actually increase atherosclerosis as illustrated by studies of modified pregnancy-associated plasma protein A (PAPP-A) expression (350, 722). PAPP-A is a metalloproteinase that degrades IGFBP-4 (IGF-I binding protein 4), thereby increasing local IGF-I availability and signaling. (There are 6 IGFBP proteins, with various controls and feedback mechanisms, bringing substantial complexity to IGF-I signaling.)

Another growth factor, PDGF, provides only limited additional insights. PDGF signaling clearly promotes postinjury neointimal formation, but its role in atherosclerosis is am-
binds the ubiquitin ligase Cbl and may also modulate Cbl
Sos, and ERK activation (1256, 1296, 1744). LRP1 also
and PI3K activity), followed by binding of ShcA, then Grb2-
cytoplasmic tail of LRP1 (possibly with assistance from Src
1296). Activated PDGFR
endosomes after internalization of the two receptors (1256,
clearly accelerated atherosclerosis (193). Importantly,
LDL seems to stabilize LRP1 and increase its expression by
decreasing ubiquitin-mediated degration (240). VSMC-spe-
cific deficiency of LRP1 in LDLR-null mice resulted in thick-
ening of the media, dilation of the aorta, aneurysm forma-
tion, and accelerated atherosclerosis (191). Importantly,
feeding VSMC-specific LRP1-deficient mice a high-fat,
high-cholesterol diet led to dramatically accelerated athero-
sclerosis with massive foam cell formation and occlusion of
mesenteric vessels and even aortic occlusion followed by
death of the animals within 2 mo. LRP1 can bind at least 40
ligands and modulates signaling of other receptors. In liver,
more inflamed or activated in the PDGF-B-null chimeric
mice.

The LRP1 receptor has major effects on VSMCs. Interest-
ingly, LRP1 can mediate uptake of aggregated LDL and
subsequent cholesterol accumulation in VSMC. Aggregated
LDL seems to stabilize LRP1 and increase its expression by
decreasing ubiquitin-mediated degration (240). VSMC-spe-
cific deficiency of LRP1 in LDLR-null mice resulted in thick-
ening of the media, dilation of the aorta, aneurysm forma-
tion, and accelerated atherosclerosis (191). Importantly,
feeding VSMC-specific LRP1-deficient mice a high-fat,
high-cholesterol diet led to dramatically accelerated athero-
sclerosis with massive foam cell formation and occlusion of
mesenteric vessels and even aortic occlusion followed by
death of the animals within 2 mo. LRP1 can bind at least 40
ligands and modulates signaling of other receptors. In liver,
together with LDLR, LRP1 mediates endocytosis of TGR
into hepatocytes (192). In VSMC, LRP1 physically interacts
with and restrains the activity of both the PDGFRβ and the
tGF-β receptors. Absence of VSMC LRP1 led to increased
cell-surface PDGFRβ expression, PDGF-BB signaling, and
VSMC proliferation.

LRP1 restraint of PDGFRβ signaling in VSMC is not fully
understood. LRP1 interaction with PDGFRβ may occur in
endothones after internalization of the two receptors (1256,
1296). Activated PDGFRβ can tyrosine phosphorylate the
cytoplasmic tail of LRP1 (possibly with assistance from Src
and PI3K), followed by binding of ShcA, then Grb2-
Sos, and ERK activation (1256, 1296, 1744). LRP1 also
binds the ubiquitin ligase Cbl and may also modulate Cbl
ubiquitination of PDGFRβ and therefore its expression or
degradation (1744).

Excessive PDGF signaling in LRP1-deficient VSMC is ac-
companied by enhanced tGF-β signaling. Normally, LRP1
forms a complex with and is thought to sequester tGF-βRI,
thereby reducing signaling through the tGF-βRI/II com-
plex, diminishing phosphorylation of SMAD2. SMAD2/3
activation induces both PDGF and PDGFRβ expression
(193). Interestingly, the PPARγ agonist rosiglitazone re-
strained tGF-β signaling and decreased atherosclerosis in
mice with VSMC-specific LRP1 deficiency (193). LRP1 also
mediates endocytosis of thrombospondin-I (Thbs1) and
several active MMP. Active MMP2 and MMP9 are
increased in the aorta of mice with LRP1 deficiency in VSMC
and could contribute to aneurysm formation. Finally, LRP1
also appears to induce expression of Wnt5a which inhibits
cholesterol and cholesteryl ester accumulation in adi-
pocytes and may also be involved with cholesterol accumu-
lation in VSMC (1773).

Another LRP, LRP6, may act similarly to restrain PDGFRβ
signaling. LRP6 is considered to be a canonical coreceptor
in Wnt signaling. However, both the rare LRP6 R611C
variant and wild-type LRP6 were also found to interact
with PDGFRβ. Unlike wild-type LRP6, the R611C variant
failed to target PDGFRβ to lysosomal degradation (identi-
ced as the predominant means of degradation, rather than
via the proteosome) with a result that PDGFRβ signaling,
including ERK activation, was markedly increased in the
presence of the R611C variant. These results may also serve
to clarify the role of LRP1. Presumably, both LRP1 and
LRP6 serve in VSMC to target PDGFRβ to lysosomes for
degradation, possibly by Cbl-mediated monoubiquitina-
tion. In these studies, R611C acted as a dominant negative
inhibitor of normal LRP6 activity. These results may ex-
plain the dominantly transmitted, severe atherosclerosis
seen in a family with the rare LRP6 R611C mutation/vari-
ant (1134). Carriers of the mutant LRP6 also had moder-
ately elevated LDL-C (mean 170 mg/dl in affected versus
98 in noncarriers), mild hypertriglyceridemia, hypertension,
and diabetes, all without obesity. Premature osteoporosis
was also apparent. However, the severity of premature
CAD seemed out of proportion to these risk factors. A
remarkable 29 of 59 pedigree members had CAD with onset
prior to age 50 in men and 55 in women. Of the 29 with
CAD, 23 died of CAD at a mean age of 52, while pedigree
members without early CAD had a mean age of death of
81 (1134). This is similar to or even greater than the 10-
to 20-fold excess CAD risk seen with untreated heterozygous
FH (781). LRP6 R611C also failed to facilitate Wnt signal-
ing in VSMC, although these results could not explain the
excessive proliferation seen in vivo and in response to
PDGF (896). However, classical Wnt signaling, besides pro-
moting proliferation, may also block apoptosis of VSMC
exposed to ROS, RNS, oxLDL, and inflammatory cyto-
kines such as IL-1β and IFN-γ (1208). Whether defects in
Wnt signaling might further contribute to the pro-athero-
genic effects of LRP6 R611C and LRP1 deficiency has ap-
parently not been examined.

The second coreceptor in canonical Wnt signaling, LRP5,
has been studied in hyperlipidemic mice. Absence of LRP5
clearly accelerated atherosclerosis (a 3.5-fold increase in
aortic area involved in lesions). This increase may have been
out of proportion to the 60% increases in VLDL and

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LDL-C seen. Triglycerides after fat feeding were increased to a greater extent with evidence that TGRL removal was impaired. Nevertheless, the potential involvement of VSMC Wnt signaling in this model might be a fruitful subject for future research (1113).

Proliferating VSMC can contribute to plaque size, and VSMC may be transformed into foam cells and express an activated, secretory phenotype. Yet, their ability to produce collagen and thicken the fibrous cap helps stabilize plaques. TGF-β is both anti-inflammatory and promotes VSMC deposition of collagen. But it also promotes deposition of LDL-binding proteoglycans. The net effect of interrupting position of collagen. But it also promotes deposition of captured. VSMC stimulated simultaneously through TNFR1 inflammation in atherosclerotic lesions has recently been re-

A newly recognized contribution of medial VSMC to inflammation in atherosclerotic lesions has recently been reported. VSMC stimulated simultaneously through TNFR1 and LTβR ligation can direct lymphorganogenesis in the adventitia, resulting in a collection of activated immune cells which may contribute to the inflammation and progression of the nearby plaque (1077).

Finally, an additional, surprising feature of VSMC behavior in advanced plaques should be mentioned. Culprit plaques causing acute coronary syndromes in humans have been consistently shown to rupture just proximal (upstream) to the point of maximum stenosis. This is the area of most rapid flow (560). This is true even though the area of slow flow just downstream of the stenosis is where endothelium is activated and foam cells accumulate. It appears that while high shear stress promotes endothelial integrity and higher NO production in the upstream area, the higher shear may simultaneously promote plasmin production as well. Furthermore, NO appears to inhibit underlying VSMC proliferation and collagen synthesis as well as promote VSMC apoptosis. Why this would be the case is not clear since PKG, activated by NO, actually promotes VSMC proliferation and PKG deficiency led to smaller lesions with fewer VSMC in hyperlipidemic mice (1972). Perhaps prolonged rapid flow stimulates changes in VSMC that are analogous to the normal compensatory vessel enlargement in areas of rapid flow, particularly during embryogenesis, but in the context of an advanced atherosclerotic plaque such changes may lead to paradoxical weakening of the fibrous plaque just under the area of endothelium exposed to the highest flow rates (1641, 1642).

In contrast to TGF-β, the effect of IFN-γ from T-cells to suppress VSMC collagen production is generally considered destabilizing (1042). Additionally, localized overexpression of p53 in carotid plaques of ApoE-deficient mice led to an increase in VSMC apoptosis in the fibrous caps, thinning of the cap, and spontaneous rupture upon hemodynamic challenge with phenylephrine (1887). Activated macrophages produce FasL which binds to Fas (the so-called “death receptor”) on VSMC, promoting VSMC apoptosis (821) and plaque instability. Importantly, infusion of smooth muscle progenitor cells in ApoE KO mice decreased plaque size and macrophage content if given early in life and led to increased plaque collagen content (2121).

A newly recognized contribution of medial VSMC to inflammation in atherosclerotic lesions has recently been reported. VSMC stimulated simultaneously through TNFR1 and LTβR ligation can direct lymphorganogenesis in the adventitia, resulting in a collection of activated immune cells which may contribute to the inflammation and progression of the nearby plaque (1077).

Genetic animal models have provided many insights into molecular mechanisms leading to advanced, vulnerable plaques. Accumulation of excessive cholesterol or endocytosis of modified lipoproteins can promote inflammation in macrophages and may lead to apoptosis. If limited, apoptosis of macrophages and neutrophils occurs without necrosis. Rapid removal of apoptotic cells can be accomplished by both macrophages and VSMC through the process of efferocytosis, with perhaps limited egress of lipid-laden macrophages out of the plaque. These features are hallmarks of early, easily reversible lesions (1726). With continued recruitment of additional macrophages and other inflammatory cells combined with local proliferation (846) and lipid accumulation, the number of apoptotic cells increases and efferocytosis seems to slow or become insufficient. Cholesterol-laden macrophages are particularly prone to both impaired efferocytosis and ER stress leading to even greater accumulation of apoptotic cells. Apoptotic cells that are not phagocytosed undergo secondary necrosis, and these accumulate and promote even greater inflammation. Continuing accumulation of uncleared necrotic foam cells leads to increasing release of inflammatory cytokines and matrix-dissolving enzymes. At the same time, impaired VSMC-mediated fibrosis may lead to a thinned fibrous cap and vulnerable plaques. Interestingly, this process is similar to certain chronic infections. Indeed, advanced plaque macrophages show striking similarities to those in tuberculous granulomas (1582).

1. Macrophage inflammation through CD36 and STAT1 signaling

CD36 has been listed as one of the major scavenger receptors that, on macrophages and probably VSMC, can mediate uptake of various modified lipoproteins including ox-LDL, AcLDL, VLDL, and TGRL remnants. CD36 also recognizes specific oxidized phospholipids. CD36 is not a passive player in the process of lipoprotein uptake. The cytoplasmic tail of CD36 forms a complex with paxillin and the Src family kinase Lyn. Upon ligand binding to CD36, Lyn activates PYK2 which tyrosine phosphorylates p130CAS leading to downstream activation of JNK1/2 and ERK1/2 together with ROS production (716, 1691). Furthermore, active Lyn tyrosine phosphorylates CD36 which promotes formation of a CD36:TLR4:TLR6 complex...
which binds MyD88 with downstream signaling to activate NF-κB which increases production of pro-IL-1β and various chemokines. TLR4 further binds the adapter TRIF with increased production of type I interferons, IFN-α/β (1684). CD36 can also form a complex with TLR2 with further activation of NF-κB. Src family kinases can also mediate signaling through a PLCγ-DAG, IP3-calcium release-PKC activation pathway, further promoting PYK2 activation. Src family kinases simultaneously activate the GEF Vav1 to exchange GTP for GDP on Rac1. Vav1 and Vav3 couple with CD36 and are required for foam cell formation upon incubation with oxLDL (1449). Active PKC and Rac1 promote assembly and activation of NOX2 to enhance ROS production (1210). These pathways provide greater understanding of the marked reduction in atherosclerosis after CD36 KO or pharmacological CD36 inhibition in mice (716).

In atherosclerotic plaques, macrophages can be exposed to substantial quantities of IFN-γ whose receptor, IFNGR2, is coupled to JAK2-STAT1. After being phosphorylated at Tyr701 by JAK2, STAT1 dimerizes and translocates to the nucleus. However, nearly 80% of the transcriptional activity of STAT1 depends on a second phosphorylation at Ser727 which leads to sumoylation at this site by Pias, STAT1 stabilization, and increased transcriptional activity (112). This serine phosphorylation can be accomplished by CaMKII in response to elevations of calcium. In macrophages, calcium elevations occur through multiple pathways, including TLR2/4 and CD36 binding as well as ER stress, providing the calcium signals and increased ROS to activate CaMKII and instigate STAT1 Ser727 phosphorylation. Macrophage STAT1 deficiency dramatically decreased apoptosis in advanced lesions as well as the accumulation of apoptotic debris in LDLR KO mice (15, 1047). Modified lipoproteins, such as AcLDL, can lead to both cholesterol overloading and CD36 ligation and are therefore especially toxic. Indeed, oxidized phospholipids binding to CD36, saturated fatty acids, oxLDL, and the lipoprotein Lp(a), which is particularly enriched with oxidized phospholipids, can all activate CaMKII leading to increased STAT1 signaling. In macrophage foam cells that are already stressed from cholesterol overloading and attendant ER stress, activation of CD36 or pattern recognition receptors such as the TLR can provide a “second hit” that results in apoptosis (1210, 1729). Activation of PYK2 through CD36 signaling will further increase the overall inflammatory response (716).

2. Free cholesterol and the NALP3 inflammasome

The toxicity of excess unesterified or free cholesterol (FC) is of key importance in advanced lesions (1645, 1725). The active macrophage, imbibing large amounts cholesteryl ester (CE) and some FC from modified LDL, TGRL remnants, and phagocytosed cells, faces a major threat from within. Foam cells can accumulate fairly large amounts of cytoplasmic CE and triglyceride with seeming impunity. However, if FC release from CE hydrolysis exceeds CE formation, FC accumulates and can trigger apoptosis or necrosis. Thus deficiency (or pharmacological inhibition) of ACAT1 increases atherosclerosis (see TABLE 8). Surprisingly, however, hydrolysis of CE is one of the rate-limiting steps of cholesterol mobilization from macrophages to HDL and inhibition of neutral cholesteryl ester hydrolase 1 (NCEH1) or hormone sensitive lipase (HSL, also abbreviated as LIPE) or both promoted foam cell formation and atherosclerosis (1584).

In addition to potentially generating FC intracellularly, macrophages avidly phagocyte cholesterol crystals as well as uric acid crystals. These preformed crystals are a much greater threat than membrane-associated free cholesterol since they can trigger the NLRP3 inflammasome with resulting IL-1β secretion as shown in FIGURE 16 (452, 1453). Briefly, cholesterol crystals activate the intracellular pattern recognition receptor NLRP3 (NOD-like receptor family, pyrin domain containing 3) to promote assembly of the inflammasome with the adaptor protein ASC [apoptosis-associated Speck-like protein containing a CARD (caspase-associated recruitment domain)] linking NLRP3 to pro-CASP1 (caspase 1). This complex generates active CASP1 which can cleave pro-IL-1β to active, inflammatory IL-1β. Calcium mobilization also was shown to be important for NLRP3 activation (1254). Essentially all elements of the NLRP3 inflammasome were pro-atherogenic in various mouse studies (see TABLE 9). Remarkably, the transcription factor Nrf2, essential for expression of antioxidant genes, was also required for NLRP3 activation by cholesterol crystals in macrophages, possibly explaining the surprising reduction in atherosclerosis with Nrf2 KO (546). Several additional miscellaneous genes that may promote inflammation in relation to lipid accumulation are also listed in TABLE 9.

3. ER stress in foam cells

For years it has been known that macrophages can mitigate the potential toxicity of FC by stimulating phosphatidylycerine (PC) synthesis with formation of cellular whorls consisting of FC and PC. However, if the molar ratio of FC:PC remains above 0.4, necrosis ensues (1728, 1730). These older observations may help explain the intriguing finding that infusion of cholesterol acceptors, such as PC or phospholipid micelles, can cause remarkably rapid regression of atherosclerotic plaques (1962). Increased PC synthesis by macrophages in response to excess FC has come to be recognized as part of the unfolded protein response (UPR). Some elements of the UPR are depicted in FIGURES 16 AND 17. Excellent reviews of the UPR in relation to cardiovascular disease are available (1213, 1505). Briefly, in unstressed cells, three key transmembrane sensors (IRE1, PERK, and ATF6) extend through the ER...
membrane and into the ER lumen where they bind plentiful chaperones that promote protein folding. One such chaperone is BiP (immunoglobulin heavy chain-binding protein, also known as Hsp70 for “heat-shock 70-kDa protein 78” or Grp78 for “glucose-regulated protein 78”). ER stressors include excess protein synthesis, dysregulation of ER calcium, and excess or inadequate oxidative stress. An oxidizing environment in the ER is required for the disulfide bond formation that is part of proper folding for many proteins. Such disulfide bonding of nascent proteins is facilitated by protein disulfide isomerase/oxidoreductase (PDI), one of several similar proteins. To be active, PDI must be in an oxidized form and after assisting in formation of disulfide bonds it becomes reduced. Oxidized PDI is reoxidized by H₂O₂ generated by ERO1α (ER oxidoeductin 1α) which utilizes GSSG and generates 2 GSH and H₂O₂ in the process.

FC loading stiffens the ER membrane which appears to impair proper function of SERCA2b (513, 1039). Saturated fat, particularly palmitic acid, has a similar effect while unsaturated fats are protective (484, 830). One or more of the key UPR sensors in the ER membrane (PERK, IRE1, AFP6) seem also to directly sense disordered lipid composition of the ER membrane, an effect that was additive but independent of protein misfolding (1608). Steroyl-CoA desaturase 1 (SCD-1) converts palmitate or stearic acid to unsaturated fatty acids and also has a protective effect against ER stress. Fatty acid binding proteins FABP4 (also known as aP2) and FABP5 are induced by JNK/c-Jun and

FIGURE 16. Proinflammatory lipid-mediated signaling and efferocytosis in macrophages. Nrf2 is in some way permissive for activation of the NLRP3 inflammasome and IL-1β production. ACAT1, acyl-CoA:cholesterol acyltransferase 1, also known as SOAT1 for sterol O-acyltransferase 1; CE, cholesterol ester; FC, free cholesterol; nCEH, neutral cholesteryl ester hydrolase; PL, phospholipid; PS, phosphatidylserine. Src refers to Src or other Src-family protein tyrosine kinases and may include Src, Lyn, Fyn, Lck, Yes, Fgr, Hck, Blk, or Csk-1.
appear to deliver both FC and saturated fat to the ER, promoting ER stress. Additionally, these FABP, in some way, block desaturation of palmitate by SCD-1 and block the protective activation of LXRs, possibly by direct physical interaction with and inhibition of PPARγ or by binding and sequestering away natural ligands of PPARγ. KO of FABP4 leads to greater availability of these ligands, greater stimulation of PPARγ, and suppression of atherosclerosis (1123). FABP5 deletion was also protective (95, 484, 800) (TABLE 9).

Under ER stress, excess protein production or inadequate calcium can lead to over-utilization of chaperones, inadequate PDI activity, or other dysfunction of the protein processing machinery. Proteins misfold, chaperones become scarce, and their absence triggers activation of IRE1, PERK, and ATF6. PERK phosphorylates and inhibits eukaryotic translation initiation factor 2α (eIF2α), which releases the transcription factor ATF4, freeing it to induce the transcription factor CHOP (C/EBP homologous protein). Phosphorylation of eIF2α by PERK also generally downregulates other protein synthesis, a compensatory check to protect the cell from the stress of protein overproduction. Activated ATF6 also induces CHOP.

CHOP production is of particular importance as increased levels push the cell toward apoptosis or a desperate attempt to recover protein synthetic capacity. Thus CHOP induces increased expression of pro-oxidant ERO1α, pro-apoptotic Bim and CHAC1, and GADD34 while acting as a corepressor to reduce expression of anti-apoptotic Bcl-2 and Bnip-3 (1213, 1250). GADD34 is a phosphatase that targets and reactivates eIF2α, allowing stressful protein synthesis to continue. In the setting of ER stress, increased ERO1α can increase ER oxidative stress sufficiently to promote calcium loss through the oligomerization of Bax and Bak in the ER membrane, opening of IP3R and inactivation of SERCA (435, 1729, 1731). CHOP-induced calcium loss from ER can lead to excess calcium uptake by mitochondria, increased ROS production by mitochondria, and mitochondrial damage which can help activate the NLRP3 inflammasome (1254). Oxidative inhibition of SERCA can be counteracted by AMPK. This may be at least one reason why AMPK deficiency increases atherosclerosis (TABLE 1).

Some UPR signaling results in an adaptive increase in the production of certain select proteins such as chaperones, cellular machinery for endoplasmic reticulum-associated degradation (ERAD), and antioxidant genes (through Nrf2). However, continued or severe ER stress with unsuccessful adaptation results in variable degrees of inflammatory signaling including activation of NF-κB and JNK through activated IRE1 (see FIGURE 16). Eventually, apoptosis can ensue. Apoptosis can be triggered by multiple pathways, including JNK phosphorylation and inactivation of Bcl-2, caspase-12 activation, and by excessive calcium release from the ER. Besides stimulating the NLRP3 inflammasome as noted above, ER calcium escape can activate CaMKII leading to serine phosphorylation of STAT1, and activation of PYK2. ROS production by mitochondria as well as Rac1 and NOX2 with general promotion of proinflammatory and apoptotic pathways follows (1729, 1731).

X-box binding protein 1 (XBP1) is a transcription factor whose mRNA remains unspliced until processed by activated IRE1 during the UPR. Functional XBP1 then mediates the transcription of genes that will increase PC synthesis, compensating for the excess cholesterol load. Increased PC lowers the FC:PC ratio and results in expansion of the surface area and capacity of the ER (1505, 1669). Nevertheless, overexpression of XBP1 was strongly proatherogenic in one study (2060), presumably due to other effects.

Genetic models involving studied components of UPR and apoptosis are summarized in TABLE 9. Some of their effects on atherosclerosis are complex and not easily predictable. Thus deletion of apoptosis inhibitor expressed by macrophages (AIM) greatly decreased lesion size because of increased macrophage apoptosis in early lesions. Congruently, transplant of bone marrow from animals lacking the pro-apoptotic protein Bax (Bcl-2-associated X protein) had larger lesions due to more surviving macrophages. However, macrophage-specific deletion of anti-apoptotic Bcl-2 (B-cell leukemia/lymphoma 2) did not affect lesion size, although more apoptotic macrophages were seen in plaques together with more cholesterol crystals.

Despite the dire consequences of intense or prolonged UPR, limited ER stress in early lesions, with adaptive increases in BiP, actually enhanced macrophage survival (422). Other examples of a need for balanced, modest activation have been reported, as in the limited oxidative stress required to activate Nrf2. Some NF-κB pathway activity is protective (as macrophage-specific IKK2 KO caused increased atherosclerosis) and the absence of the MAPK p38 in macrophages greatly increased apoptosis and necrosis with accelerated atherosclerosis (1583). This may also explain why KO of MAPK phosphatase 1 (MKP-1), which inhibits p38, actually decreased plaque size (see TABLE 1). The protective effect of p38 seemed to be mediated by increased PKB/Akt signaling. Consistent with this observation, genetic impairment of insulin signaling decreased PKB signaling and promoted apoptosis in macrophages (1731). In addition, release of the anti-inflammatory cytokines IL-10 and TGF-β after efferocytosis is dependent on p38 (473). Unlike p38, however, JNK2 was consistently proatherogenic in genetic models as noted previously (see TABLE 1).

Not discussed in this review are the generally cytoprotective and anti-inflammatory effects of autophagy. This process is triggered by reduced energy supplies to the cell but also by many of the same cellular stress pathways (such as ER stress...
and UPR) experienced by macrophages in advanced lesions. Autophagy is also thought to be of primary importance in defense against pathogens (1704). Effects on atherosclerosis of genetic models of autophagy-related genes have apparently not been reported.

4. Macrophage-dependent resolution of inflammation: an optimal outcome of efferocytosis, PPAR, and LXR signaling

Efferocytosis (which literally means “carrying the corpse to the grave”) is a process whereby phagocytes are attracted to apoptotic, necrotic, or damaged cells followed by highly controlled engulfment and endosomal/lysosomal digestion of the effete cell. Besides macrophages and DC, endothelial cells, VSMC, and other cells can be recruited to assist in the task of cell corpse removal. Initial phagocyte attraction occurs by a host of “find me” chemoattractant signals (1411) of which several have been examined in various atherosclerosis models. These include fractalkine which binds its receptor CX3CR1 on macrophages, LPC (released by a calcium-independent isoform of PLA, designated iPLA2β) binding its GPCR GRA, and SIP. Microblebs released by apoptotic cells also attract surrounding phagocytes. Necrotic cells can additionally release separate “danger signals,” such ATP (when released at high concentration) binding to high-affinity P2Y2 and lower affinity P2X receptors, various heat shock proteins, mRNA and other RNA, and a number of other products of necrotic cells (1411). The DNA binding protein high-mobility group box protein 1 (HMGB1) is also released by necrotic cells. It acts to both attract and activate surrounding phagocytes and can activate endothelial cells, possibly by interaction with the RAGE receptor as well as TLR2 and TLR4 (1411). Administration of HMGB1 blocking antibodies decreased atherosclerosis in ApoE KO mice (878).

After being summoned, the phagocyte must encounter appropriate “eat me” signals displayed by the apoptotic cell (such as phosphatidylserine and various opsonin-like proteins) which match a series of receptors on the phagocyte, thus forming a synapse between the phagocyte and the target cell. Certain inhibitory “don’t eat me” signals, generally displayed on healthy cells, can strongly block efferocytosis. In advanced plaques there appears to be an over-abundance of efferocytosis signals with many awaiting apoptotic cells, suggesting saturation of efferocytosis mechanisms (1791, 1863). Several efferocytosis promoting ligands and receptors, together with subsequent intracellular signaling, are discussed below and are reviewed in TABLE 9 and depicted in FIGURE 17.

Virtually all of the defects of efferocytosis listed in TABLE 9 (as well as those related to Treg function) promote autoimmune disorders as well as atherosclerosis (473, 2120). Interestingly, while Fas (fragment, apoptosis stimulating) is often referred to as the death receptor, Fas, and its ligand FasL, are also among the receptors and ligands that control efferocytosis. Mice that lack functional Fas or FasL have impaired ability to clear apoptotic debris and display features of autoimmune disease. When crossed with ApoE-deficient mice, these mice have increased atherosclerosis (73, 517).

Recently, actual phagocytosis of the apoptotic cell was shown to be strongly influenced by uncoupling protein 2 (UCP2). A decrease in the mitochondrial electron gradient or mitochondrial potential was shown to be required for efficient efferocytosis while a high gradient was inhibitory (1380). Why this should be the case remains to be demonstrated. Nevertheless, impaired efferocytosis may be an additional explanation for the increased atherosclerosis seen in UCP2 KO mice (1240) as well as the accumulation of macrophages present in lesions of mice receiving UCP2 deficient bone marrow (157).

Efferocytosis can provide macrophages with a large influx of lipids that can act as PPAR and LXR ligands, as noted above. Besides increasing cholesterol flux through macrophages, lipid-bound LXR and PPAR nuclear receptors are directly and profoundly anti-inflammatory. Most recently, a remarkable insight regarding the key LXR ligand in foam cells was presented. In the setting of increased cholesterol content, SREBP2-mediated synthesis of cholesterol is downregulated. However, 24-dehydrocholesterol reductase (DHCR24) is suppressed to <5% of its full activity, while hydroxy-methyl-glutaryl CoA reductase (HMGCR) retains ~30% of its activity. DHCR24 converts desmosterol to cholesterol. The resulting imbalance between HMGCR (and other components of the cholesterol synthesis pathway) and DHCR24 is an accumulation of desmosterol. Desmosterol, in turn, was found to be a key ligand for LXR. Surprisingly, desmosterol was found at concentrations 20–50 times those of oxidized cholesterol species that are also LXR ligands. This was true in human plaque as well. The effect of this abundance of desmosterol was a profound suppression of inflammatory signaling as a direct result of LXR signaling as depicted in FIGURE 17 (1662). Not shown were alteration in fatty acid metabolism leading to several anti-inflammatory fatty acids as well. Thus at least some degree of FC accumulation in ER membranes should actually be anti-inflammatory. These observations may explain why in human plaque up to 20% of macrophase/foam cells display the noninflammatory, resolving phenotype labeled M2 as opposed to proinflammatory M1 (1662).

One mechanism of this anti-inflammatory effect of LXR involves large corepressor complexes. In macrophages and other effector cells, nuclear corepressor (NCoR) and silencing mediator for retinoid and thyroid hormone receptors (SMRT) physically interact with NF-κB, AP1, and other inflammatory transcription factors while they are bound to
DNA, thereby preventing transcription of inflammatory gene targets (see **FIGURE 17**). Such cells are poised for rapid activation when these corepressors are released by inflammatory signals. When bound by ligand, both LXR and PPARγ can be sumoylated and, as such, interact directly with and prevent removal of NCoR and SMRT, a mechanism termed “transrepression” (286, 794, 819).

The anti-inflammatory mechanism of PPARγ is less direct. PPARγ agonists are anti-inflammatory and antiatherosclerotic in hyperlipidemic mice. Yet, bone marrow transplant with PPARγ-deficient cells decreased atherosclerosis even more than did PPARδ agonists (108, 988). Resolution of this apparent paradox came when investigators found that nonliganded PPARγ binds and holds inactive the powerful nuclear corepressor Bcl6 (B-cell lymphoma 6). Thus PPARγ is anti-inflammatory essentially only when ligand binding causes release of Bcl6. When freed from PPARγ, Bcl6 acts as a corepressor to repress transcription. Mice lacking TSC1/TSC2 in inflammatory genes

**FIGURE 17.** Anti-inflammatory lipid-mediated signaling and efferocytosis in macrophages. Nuclear receptor corepressor 1 (NCoR) functions as an inhibitory deacetylase complex. An important anti-inflammatory function of LXR and PPARγ is to block the removal of NCoR from the promoters of proinflammatory genes. LPC binding to GPCR G2A may promote efferocytosis. Not shown is extensive anti-inflammatory signaling that occurs with APOA1 binding to ABCA1. PC, phosphatidylserine; FFA, free fatty acids; LPC, lysophosphatidylcholine; LXA4, lipoxin A4; Ma, maresins; PD1, protectin 1; RvD/E, resolvins of the D and E series; S1P, sphingosine-1-phosphate.
Bcl6 have hyperinflammatory responses to LPS and increased atherosclerosis (109, 110). PPARα also has direct anti-inflammatory effects, but these mechanisms appear less well defined (286).

LXR and PPARδ also orchestrate prompt efferocytosis by inducing genes involved in sensing “eat me” signals, secretion of proteins with an opsonizing effect, and production of anti-inflammatory cytokines including IL-10. In addition to LXR and PPAR effects, efferocytosis itself initiates other anti-inflammatory signaling. Numerous studies demonstrate rapid downregulation of inflammation upon first contact with early apoptotic cells (1431, 2120) or cell fragments (466). Some of this anti-inflammatory effect is mediated by phosphatidylserine receptors. For example, ligation of MerTK (Mer receptor tyrosine kinase) led to rapid inhibition of NF-κB signaling through activation of the PI3K/PKB pathway (466). MerTK is induced by LXR and utilizes Gas6 as a bridge to phosphatidylserine (286). Another phosphatidylserine receptor on macrophages is the αvβ3 integrin which utilizes milk fat globule-EGF factor 8 (Mfge8) or transglutaminase 2 (TG2) as bridging molecules. Genetic deficiency of most of these mediators of efferocytosis increased atherosclerosis and the number of apoptotic and necrotic cells in plaques (TABLE 9).

While generally depicted as a means of promoting cholesterol efflux from macrophages, ApoE secreted by either the phagocytosing macrophage or apoptotic macrophages promotes efferocytosis and downregulates inflammatory responses. These effects are mediated by binding to LDL receptor-like protein 1 (LRP1) which, in this setting, activates cAMP, PKA, and PKB signaling followed by greatly reduced responsiveness to TNF-α (2016).

An influx of lipids from ingested lipoproteins and efferocytosis may also promote synthesis of lipoxins (such as LXA4), resolvins, protectins, and maresins with marked anti-inflammatory effects, but these mechanisms appear less well defined (286).

The lipoxins were identified first and are produced from arachidonate. E-series resolvins are produced from EPA and D-series resolvins from DHA. Maresins are produced by macrophages and are the most recently identified of these resolving eicosanoids. Importantly, their actions on specific GPCR include inhibition of neutrophil diapedesis (thus signaling to end the acute phase of inflammation), decrease in edema formation, antagonism toward TNF-α and NF-κB signaling, and yet they are chemoattractant toward macrophages while stimulating them to take on an M2 phenotype to promote prompt resolution of inflammation and removal of apoptotic cells and cellular debris (544, 1590, 1591). Acting through the GPCR ChemR23, for example, the resolvin E1 stimulated Gαi-mediated activation of PI3K/ PKB followed by PKB, mTOR, p70S6K, and ribosomal S6 phosphorylation, which were all required to support increased macrophage phagocytosis (544).

Surprisingly, acetylation of COX-2 by aspirin, rather than completely inactivating the enzyme, converts COX-2 to a 15-lipoxygenase-like enzyme, synthesizing 15R-hydroxyeicosatetraenoic acid (15R-HETE) from arachidonic acid. The product of native 15-lipoxygenase is the chiral alternative, 15S-HETE. Both 15S- and 15R-HETE are substrates for 5-lipoxygenase to produce highly anti-inflammatory, pro-resolving lipoxins. Furthermore, the aspirin-triggered lipoxins are more resistant to metabolic inactivation. In addition, the modified COX-2 enzyme could support production of resolvin precursors from n-3 fatty acids such as DHA (1330, 1593). Adenosine, potentially encountered in apoptotic cells, can act through the purinergic receptor A3R to block IFN-γ-mediated activation of STAT1 (112).

In addition to anti-inflammatory signaling triggered by efferocytosis, phagocytes may encounter many activation or “danger” signals in apoptotic and especially necrotic cells. Thus the prior history of an apoptotic cell, including microbe infection, previous ligation of TLRs, cholesterol loading, an encounter with cholesterol crystals, or in one study, prior exposure to oxLDL (1280), can modulate the effect of its subsequent phagocytosis by another cell from anti-inflammatory to pro-inflammatory (1431, 2120). Indeed, one can imagine that the complex array of proinflammatory triggers encountered deep within an actively growing atherosclerotic plaque could lead to repeated cycles of apoptosis and necrosis followed by toxic or proinflammatory efferocytosis, converting the necrotic core into an immunological “no-man’s land.”

**D. The Complement Pathway and Atherogenesis**

The complement pathway was so named for its ability to complement the action of antibodies in lysing bacteria. It consists of a series of serine proteases or convertases (called zymogens in their inactive, uncleaved form), with each activated protease capable of lysing and thereby activating...
many copies of its target zymogen, resulting in an amplifying cascade that ends with the production of many copies of active C9. Active C9 multimers are inserted through the cell membrane to form numerous large pores causing osmotic lysis of the targeted cell. Several of the lytic pieces generated by zymogen activation are inflammatory or attack to cells and target them for phagocytosis, a process called opsonization. The classical, alternative, and lectin pathways lead to activation of the key C3 and C5 convertases. Host cells carry inhibitors and regulators of the pathway to minimize peripheral damage. Briefly, for classical activation, C1qa (complement component 1, q subcomponent, A chain) recognizes and binds the Fc portion of antibodies bound to antigen, commonly on the surface of an invading bacterium. C1q exists as a hexamer that looks like an inverted umbrella, with a globular head at the tip of each of its radiating spokes that binds targets. C1q is joined by two copies each of C1r and C1s to form the C1 complex. C1q bound to at least two separate Fc regions activates the protease C1r which then cleaves and activates C1s. C1s cleaves C4 and C2 with resulting C4b and C2a forming a protease to cleave C3. Cleavage of C3 and C4 exposes an internal, reactive thioester bond which covalently binds nearby membrane polysaccharides or proteins, with hydroxyls or amines, respectively, displacing the sulfur in the bond. In an aqueous environment such as plasma, the thioester bonds are merely hydrolyzed, and the components are inactivated. C3b, now covalently bound to the membrane joins with C4bC2a to form a C5 convertase with C5b as its product. C5b then assembles C6, C7, and C8 with insertion of C7 and C8 into the cell membrane followed by assembly of C9 multimers and pore formation. Factors C5b through C9 are together referred to as the membrane attack complex (MAC).

Emerging understanding of the complement pathway in atherosclerosis points to a protective role for early components and related molecules which bind or opsonize apoptotic cells (besides invading bacteria) and promote effecrocytosis. In contrast, assembly of later components that promote cell damage are atherogenic (1663). These findings are summarized in Table 9. Only a few orienting comments will be given here.

Penetratin 3, long pentraxin (PTX3) and C-reactive protein (CRP) are both acute phase reactants of the pentraxin family which act as part of the innate immune response to coat or opsonize invaders such as certain bacteria. They are capable of subsequently triggering the classical complement pathway when the opsonized invaders encounter the first component of the complement pathway, C1q. Thus, in addition to the Fc portion of antigen-bound antibodies, C1qa also recognizes and binds PTX3, CRP, the phosphatidylserine on apoptotic cells, and the membrane of certain bacterial cells, and can thereby activate the classical complement cascade.

Besides activating the classical complement pathway, “invaders” coated with IgG or IgM antibodies can be phagocytosed by interacting with Fcγ receptors, or when C1q is bound by the calreticulin/CD91 complex on phagocytes (1431, 1663). Apoptotic cells opsonize by C3b and C4b can be phagocytosed through recognition by other macrophage receptors (1663). C1q KO was atherogenic (Table 9). Importantly, both PTX3 and CRP can also bind apoptotic cells and promote effecrocytosis. CRP in particular recognizes oxidized phospholipids. PTX3 appears to be anti-atherosclerotic while most CRP manipulations resulted in little or no change in atherosclerosis. The few significant effects that were seen for CRP were consistently protective (see Table 9).

C1q is also activated by enzymatically modified LDL containing free fatty acids together with phospholipid (151). C1q enhanced the uptake of oxLDL into macrophages with subsequent activation of the cells including increased CD80, PECAM1, and MCP-1 release but also induction of ABCA1 and ABCG1 with enhanced cholesterol efflux to HDL (543). Remarkably, adiponectin has an analogous structure to C1q, also opsonizes apoptotic cells and promotes their effecrocytosis, binding to the same calreticulin/CD91 receptor complex as C1q (1746). It should be noted, however, that the main action of adiponectin appears to be activation of ceramidase through binding its receptors AdipoR1 and AdipoR2, thereby causing the deacetylation of ceramide with downstream formation of the anti-apoptotic S1P (778). Overexpression of adiponectin decreased atherosclerosis (1339).

The alternate complement pathway is triggered when C3b, continually produced at a low level, is activated when it covalently binds apoptotic cells, enzymatically modified LDL, cholesterol crystals or other atherosclerotic debris, and other foreign surfaces (1663). Active C3b then binds CFB (complement factor B) which is cleaved by factor D to produce the alternative pathway C3 convertase consisting of Bb and C3b. When BbC3b is joined by another C3b, it becomes a C5 convertase. The alternative pathway thus serves to amplify the classical pathway. Interestingly, no effect of CFB KO was seen when mice were fed a low-fat diet. During a high-fat diet, LDL-C was much lower in CFB KO mice which likely explained the modestly decreased atherosclerosis. However, CFB KO mice were almost entirely protected from an accelerated atherosclerosis that was induced by chronic intraperitoneal LPS administration (1124). These results demonstrate that enhancement of atherosclerosis by LPS or chronic infection need not be mediated solely by Toll-like receptors but that alternative complement pathway activation can also contribute.

Mannose- or mannan-binding lectin (MBL) is an initiating factor in the lectin pathway of complement activation with a structure highly analogous to C1q. It can coat and pro-
mote killing of certain bacteria and viruses by activating the classical complement cascade much like C1q. In human epidemiological studies, associations with CAD have been inconsistent (374, 893, 1110, 1331, 1527). More recently, MBL was recognized to opsonize apoptotic cells and other debris and facilitate effecytosis. MBL can also enhance macrophage ingestion of oxLDL as can C1q (543). MBL was found to be atheroprotective in LDLR KO mice (see Table 9). Relevance of MBL for human disease continues to be debated however (743).

Complement activation downstream of C3 is generally proatherosclerotic. This was particularly apparent for factor C6 and CD59 (see Table 9). CD59 inhibits C9 and formation of the pore-forming MAC. Lesions in CD59 KO mice had more macrophages and T-cells, more apoptosis, larger necrotic cores, decreased fibrous tissue, and much thinner fibrous caps, all leading to frequent coronary occlusion (in 73% of CD59 KO vs 9% of controls). These CD59 KO mice had decreased overall survival as well. Excess atherosclerosis was blocked in these mice by an antibody directed against C5. Furthermore, lesions were significantly decreased in mice with transgenic overexpression of CD59 primarily in endothelium, an effect also blocked by antibodies against C5 (1985). The protective effects of CD59 were confirmed by another group (1017), although more equivocal results were reported by others (60, 2052). These animal studies are consistent with an epidemiological study finding mildly increased risk of cardiovascular events in those having higher plasma levels of soluble C5b-9 as evidence of an activated complement system (1189). Factors earlier in the pathway were less strongly or nonsignificantly associated with risk (479). However, C3 is an acute phase reactant (1233, 1406), while synthesis of C2, C4, and factor B are upregulated by IFN-γ in fibroblasts and macrophages (960, 1567). Thus plasma levels may reflect inflammation generally rather than any causal relationship and need to be interpreted cautiously.

E. HDL Supports Innate Immunity

Although atherosclerotic lesions have been documented in Egyptian mummies and even hunter gatherers (50, 51, 1787), CAD as a leading cause of death for a large segment of the population is likely a modern phenomenon. Additionally, CAD generally claims its victims well after procreation, even among strongly predisposed persons. Homozygous FH may be an exception, but a high prevalence of heterozygous FH persists nonetheless. Most other mammals show little if any atherosclerosis in a natural setting. Thus little or no evolutionary pressure toward reducing the development of atherosclerosis is likely to have ever been exerted. Why then do we have HDL? The inverse association between CAD risk and serum concentrations of HDL-C or its major structural protein, APOA1 is incontrovertible (420), yet the biological “purpose” of HDL is manifestly not simply to prevent atherosclerosis.

Proteomic analysis of HDL increasingly points to a complex array of proteins that are involved in innate immunity and related functions such as complement factors, proteins related to thrombosis, hemostasis, growth factors, and other peptides (1479). Many of these proteins are found at levels of fewer than 1 molecule per HDL particle. A subset of HDL (found in humans, apes, and Old World monkeys) which contains both haptoglobin-related protein and apoL-1 constitutes “trypanosome lytic factor 1,” an important innate defense against certain trypanosomes (1619). Besides mobilizing cholesterol, apo A-1 seems to adsorb and neutralize the inflammatory effects of LPS (748), a property of HDL that likely has functional anti-inflammatory consequences (1719). The ability of HDL to remove specific oxidized phospholipids appears to facilitate innate immune responses in macrophages (357).

HDL modulates cellular responses to inflammatory signals in a general way, since a reduction in membrane cholesterol concentration mediated by HDL can reduce signaling through many receptor complexes associated with lipid rafts including several cytokine receptors (1318). For example, HDL can suppress hematopoietic stem cell proliferation stimulated by GM-CSF acting through its receptor (2053), and it normalizes neutrophil activation caused by ApoE deficiency by mobilization of cholesterol and a decrease in lipid raft formation (1260).

The toxic effects of excess free cholesterol were discussed above. The cholesterol mobilizing effects of HDL may well support normal or continuing function of macrophages which are obliged to engulf great quantities of cholesterol during effecytosis of apoptotic and necrotic cells. Such support may be critical in the setting of infection resolution. Thus the parallels between the benefits of HDL in atherogenesis may be coincidental to the role of HDL in infection or other inflammation. HDL has been noted to be particularly important after effecytosis of previously inflamed foam cells (742) (possibly because HDL can mobilize oxidized cholesterol), but such cells may arise in the setting of chronic intracellular infection, as with Mycobacterium tuberculosis (1582), as well as during atherogenesis.

HDL appears to have an effect on common infectious illness as well. In these studies it is important to note that HDL is acutely lowered by various cytokines produced in response to certain illnesses or infections. Thus studies reporting worse outcomes associated with low HDL in already infected patients (or in those with certain cancers) may provide little insight into potentially causal associations (664). Nevertheless, some data do suggest benefit with higher HDL with regard to infectious disease. Mortality from infection was 35% lower in men 85 and older with higher HDL (128). Risk of nosocomial and surgical infections were higher in persons with low HDL (251). Risk of noso-
comial respiratory infection was reported to be increased 10-fold in patients with very low HDL (<20 mg/dl) (406).

1. Emphasis on the role of HDL in innate immunity may clarify observations regarding reverse cholesterol transport

Reverse cholesterol transport is often considered among the most important functions of HDL to limit atherogenesis (401, 1232, 1290). Severe atherosclerosis has long been associated with a variety of APOA1 mutations (but not all) that result in APOA1 deficiency in both human and animal studies (990, 1548, 1637). Mice are clearly protected by over-expression of APOA1, and there is rapid regression of atherosclerosis when native or other forms of APOA1 or various AI mimetics are infused. Later, SR-B1 was recognized to be involved in HDL-mediated cholesterol delivery to the liver and interactions with other cell types. Severe, fulminant atherosclerosis and coronary lesions with appearance similar to human disease develop in ApoE-deficient mice lacking the SR-B1 receptor (204). As discussed below, SR-B1 effects other than selective cholesterol ester uptake from HDL into hepatocytes may be key to understanding the antiatherogenic effects of SR-B1.

Two studies have recently examined cholesterol efflux capacity of plasma as a predictor of CAD in humans (902, 1038). Both studies utilized plasma from the test subjects that had been treated to remove APOB-containing lipoproteins. This treated plasma was then diluted (2–4%) and incubated with macrophages that had been previously loaded with radiolabeled FC. The FC loading was done in conditions such that essentially no radiolabeled CE was formed. In both studies, greater efflux capacity of subject plasma (the amount of radiolabeled FC transferred from the macrophages to the diluted plasma) was strongly associated with reduced risk of CAD in case-control (cross-sectional) analyses. This efflux capacity of plasma was more closely associated with CAD than with HDL-C or even APOA1 itself. In addition to HDL-C, the efflux capacity in this assay was strongly dependent on the presence of precursor HDL particles, pre-β-1 HDL, which are the primary cholesterol/phospholipid acceptors via ABCA1 (395). In the second study (1038), investigators examined how the radioactive FC label distributed to different plasma components. Only ~30% of the labeled FC was found in HDL particles. About 45% was associated with APOA1 as either free or possibly pre-β HDL (but not in the HDL density range). Much of the remainder was bound to albumin, demonstrating a significant nonspecific transfer of cholesterol from the macrophages. Also in the second study, data were available for 3-year incidence of new clinical coronary events within a large angiographic cohort which was followed prospectively. The incidence of new coronary events was paradoxically higher among those with greater plasma efflux capacity (even though cross-sectional analyses in the same group at baseline and in a separate community case-control group showed an inverse association). Details regarding treatment of these higher risk subjects after the test plasma was collected were not provided. Subsequent treatment effects in the angiographic group may well have confounded these results. However, another interpretation may be that high plasma levels of pre-β HDL frequently reflect the more rapid generation of these particles in plasma in the face of high risk elevations in triglycerides and low HDL-C through the action of CETP and/or PLTP. Indeed, pre-β HDL levels in plasma can reflect multiple competing metabolic effects (e.g., initial generation of APOA1 and precursor particles, conversion to larger particles both after interaction with ABCA1 and ABCG1 and via the LCAT reaction, regeneration of pre-β HDL by CETP- and/or PLTP-mediated speciation of triglyceride-enriched spherical HDL, interactions with hepatic and endothelial lipases, renal loss, and transfer into the interstitial fluid space). Perhaps not surprisingly, in other studies using different methods to directly measure plasma pre-β HDL, associations with CAD ranged from significantly positive to significantly negative (733, 1221, 1508). Therefore, in some settings, higher pre-β HDL plasma concentrations may manifest an indirect association with increased CAD risk even though pre-β HDL itself appears to be mechanistically protective.

Prior discussion in this review emphasized the notion of reverse cholesterol transport as a means to harmonize a number of otherwise disparate observations such as effects of LCAT and PLTP on atherogenesis. However, a greater focus on the role of HDL in its interactions with cells of the innate immune system, including endothelial cells, might provide greater clarity. Mobilization of excess free cholesterol should promote survival (allowing more effective and continuing efferocytosis) and limit the activation of macrophages and probably VSMC. How or where HDL rids itself of the acquired cholesterol may be of less importance, the only significance of this unloading step being regeneration of active cholesterol acceptors and preservation of the number of APOA1-containing particles to carry out their varied tasks. Such a view would not be discordant with observations on reverse cholesterol transport noted above and may help explain the negative results to date of intervention trials using CETP inhibitors (1575). If ongoing trials with the more effective CETP inhibitors are positive, it may well be that the benefits are driven by the substantial reduction in LDL-C seen with these agents (250). Such a view also reconciles misleading generalizations regarding the lack of importance of gene variants that affect HDL levels in predicting CAD risk, since these studies were strongly influenced by genes such as CETP and endothelial lipase that may primarily affect unloading steps (1883). Clearly, certain APOA1 variants are associated with CAD even in population-wide resequencing studies (685), while rare APOA1 variants can cause premature atherosclerotic disease even in heterozygous form (381, 990).
Besides stimulation of cholesterol efflux from macrophages, endothelial, and other cells, HDL mediates a number of additional protective effects. In various in vitro studies, HDL is known to protect cultured endothelial cells from apoptosis induced by oxLDL, TGRL, TNF-α, and activated complement. HDL stimulates NO production by eNOS and increases PGI₂ production through COX-2. HDL suppresses expression of VCAM-1, ICAM-1, and E-selectin by endothelial cells with decreased monocyte adherence, as well as reducing other signs of endothelial activation after exposure to oxLDL or cytokines (1315). HDL may also inhibit monocyte activation after interacting with T-cells (1338).

2. ABCA1 is a signaling hub as well as a cholesterol and phospholipid transporter

In addition to its role as a cholesterol/phospholipid transporter, ABCA1 supports a remarkable array of anti-inflammatory intracellular signaling when bound by APOA1 (1349). In BMT experiments, lack of macrophage ABCA1 increased atherosclerosis in LDLR-deficient mice (1849). Overexpression of ABCA1 on endothelium led to both a reduction in atherosclerosis and an increase in HDL in C57Bl/6 mice fed an atherogenic Paigen diet (1838). A brief summary of the control of ABCA1 expression and anti-inflammatory signaling mediated by ABCA1-APOA1 interaction follows.

About 80% of plasma HDL is thought to be produced by the liver with the remainder released from intestine (219, 1795). In both these tissues, ABCA1 plays a critical role in ultimate release of HDL particles from the organ. Unlike apo-B-containing TGRL which are released essentially intact from hepatocytes or enterocytes, HDL is released as a precursor followed immediately by binding of lipid-poor APOA1 to the extracellular (and/or possibly the intravesicular) surface of ABCA1. Without this interaction and obtaining phospholipid and FC through the action of ABCA1, released APOA1 is rapidly cleared by the kidneys, and HDL levels are exceedingly low (as in Tangier disease).

Not only does ABCA1-induced lipid transfer stabilize APOA1, but the interaction of APOA1 with ABCA1 stabilizes ABCA1. In the absence of APOA1, ABCA1 undergoes rapid proteolysis and degradation by calpain which recognizes a proline-glutamate-serine-threonine rich (PEST) sequence in one of the cytosolic loops of ABCA1. PEST members Thr1286 and Thr1305 are constitutively phosphorylated, allowing recognition by calpain. However, binding of APOA1 leads to dephosphorylation of these threonines and stabilization of ABCA1 (1149). Without APOA1, ABCA1 half-life in the plasma membrane of macrophages was under 1 h (1149), while in intestinal cells the half-life was 10 h, presumably due to constant exposure to actively synthesized and secreted APOA1 (525).

APOA1 binding to ABCA1 led to ABCA1 stabilization by additional mechanisms. Calcium influx is also triggered by APOA1 binding to ABCA1 by an apparently unknown mechanism (885). Calmodulin is activated and binds to ABCA1 blocking calpain-mediated degradation (885). The increased calcium together with DAG produced by reactions triggered by lipid mobilization (see below) result in activating PKCa. In turn, PKCa phosphorylates ABCA1 at serine/threonine residues near the PEST sequence which inhibits proteolysis of ABCA1, resulting in increased protein expression in cell membranes (1558, 2087). Finally, APOA1 binding to ABCA1 leads to binding of PDZ-RhoGef and leukemia-associated Rho GEF (LARG) to the PDZ domain of ABCA1, activating RhoA which, in some way, acutely inhibits rapid degradation of ABCA1. Although activation of RhoA acutely protects ABCA1 from degradation, chronic RhoA activation decreases PPARγ and LXR induction of ABCA1 while prolonged inhibition of RhoA promotes ABCA1 transcription (1342).

Transcription of ABCA1 and ABCG1 is mediated by LXR/ RXR heterodimers as noted previously. ApoE appears to upregulate ABCA1 expression, supporting cholesterol efflux through an additional pathway besides direct cholesterol binding by ApoE itself (2095). Inflammatory stimuli that activate NF-κB, such as TNF-α, inhibit transcription and increase proteolysis of ABCA1 (525), providing an explanation for reduced HDL levels in conditions such as Crohn’s disease, rheumatoid arthritis, and certain cancers. Surprisingly, activation of PKCζ which accompanies APOA1 binding to ABCA1 binding through generation of DAG also promotes ABCA1 transcription through Sp1 and LXR/RXR interactions (2087). Possibly related are observations that ceramide increases expression of ABCA1 by an unknown mechanism (879). Transport of ABCA1 to the plasma membrane is facilitated by palmitoylation of ABCA1 in the ER (879). Additionally, APOA1 binding may promote direct binding of Cdc42 to ABCA1, facilitating transport of ABCA1 from trans-Golgi to the cell membrane (1065). As an example of another protein interaction, ABCA1 forms a complex with phagocytosis regulating proteins syntaxin 13 and flotillin. Syntaxin 13 helps stabilize ABCA1 while ABCA1 can promote macrophage efferocytosis (107, 526, 2039).

Some of the anti-inflammatory signaling initiated by ABCA1-APOA1 interactions is undoubtedly a result of reduced cholesterol content and fewer lipid rafts of the plasma membrane as noted above. Exactly how ABCA1 facilitates lipid transfer to HDL is still a matter of debate. According to one model, binding of lipid-free (or nearly lipid-free) APOA1 to ABCA1 (probably as a homotetramer or higher order oligomer) is thought to cause ABCA1 to actively transfer phospholipids to the outer cell membrane leaflet, causing the membrane to bulge outward (exovesiculation). This seems to occur in PC-enriched areas of the...
membrane away from cholesterol-enriched lipid rafts. The exovesiculated domain is pinched off and surrounded by APOA1 with the formation of nascent, PC-rich, cholesterol-poor HDL disks with two to four APOA1 molecules/disk (814, 1869). These PC-rich discs are particularly good acceptors of free cholesterol (FC), which is transferred to the nascent disks by diffusion from nearby lipid rafts. In other models, transfer of PC and FC to HDL occurs together and involves initial binding of APOA1 to a low-capacity binding site on or near ABCA1, followed by activation of JAK2, and transfer of the APOA1 to a high-capacity binding site followed by PC and FC loading (1065). Active ABCA1 is able to transfer cholesterol to other apoproteins with amphipathic alpha helices, including most apoproteins carried by HDL as well as ApoE (1065). Paradoxically, early cholesterol enrichment of the ER seems to promote ABCA1 ubiquitination and degradation (512), and cell membrane cholesterol enrichment impairs APOA1 binding to the high-capacity binding site on ABCA1 (814), phenomena that could both promote foam cell formation.

Sphingomyelin (SM) is also transferred to HDL in its interaction with various cells. In artificial membranes, SM is said to promote lipidation of reconstituted HDL or APOA1 (412, 564). One might postulate that this is due to SM-mediated sequestration of membrane cholesterol into the discrete, cholesterol-enriched lipid rafts, domains which remain separate from the PC-enriched areas where ABCA1 seems to partition itself. SM seems uniquely suited to this task due to its saturated fatty acid tails which effectively interact with cholesterol while the large, highly charged head allows effective interaction with the aqueous environment.

As the newly formed HDL disks adsorb cholesterol from lipid rafts, they appear to remove some SM at the same time. The lipid transporter ABCG1, which preferentially interacts with large spherical HDL, transfers SM to HDL more effectively than ABCA1 (924). Consistent with this observation, large HDL particles are greatly enriched in SM compared with small HDL (936). By whatever means, APOA1 interacting with ABCA1 and/or ABCG1 results in reduced membrane SM content. A reduction in SM can promote net transfer of phosphocholine from PC to ceramide, thereby regenerating SM and decreasing ceramide but with a net result of increased DAG. Such a reaction, together with increased calcium entry into the cytoplasm, occurs after APOA1 binding to ABCA1, the resulting DAG leading to activation of PKCa and stabilization of ABCA1 (as noted above). An inhibitor of SMS2 (and SMS1) decreased PKCa activation and also impaired cholesterol transfer to APOA1 (831, 2010).

From the above paragraph, SMS2 might reasonably be expected to be protective. However, net results from altering SMS2 activity appear to depend on cell status. It should be noted that the activity ascribed to SMS2 has been described in the literature as belonging to a putative PC-PLC, which is inhibited by the compound D609 (13). In some models, inhibition of SMS2 with D609 increased ceramide and resulted in impaired proliferation and increased apoptosis (13). In contrast, overexpressing SMS1 and/or SMS2 alongside with TNF-α treatment in Chinese hamster ovary cells or LPS treatment in macrophages led to increased apoptosis in cells while siRNA knockdown of SMS1/2 protected against apoptosis (427). In these models, SMS1/2 seemed to increase membrane SM with increased lipid raft formation and greater TNFR1 and TLR4 activity. Overexpression of SMS1/2 also suppressed HDL-mediated cholesterol efflux in this study. More recently, administration of D609 to apoE-null mice was reported to arrest progression of atherosclerosis and resulted in a more stable plaque phenotype (2077). The investigators further found that SMS2 activity appeared to be required for expression of the pro-inflammatory LOX-1 receptor in endothelial cells and that D609 treatment decreased endothelial expression of VCAM-1, ICAM-1, and MCP-1. In another study, adenoviral delivery of SMS2 to apoE KO mice increased atherosclerosis substantially but also increased non-HDL lipid levels and decreased HDL, confounding the interpretation of direct SMS2 effects (1914). Effects of these manipulations on SM in lipoproteins should also be examined since APOB-containing lipoproteins that have more SM are more subject to aggregation upon treatment with sphingomyelinase.

As noted above, the calcium influx triggered by APOA1 binding to ABCA1 promotes calmodulin binding to ABCA1 and helps prevent calpain-mediated degradation. In addition, calmodulin activation also promotes JAK2 activation in some manner that requires calcineurin (885). JAK2 apparently binds ABCA1 directly and its activation is required for normal lipid transfer activity to APOA1 and HDL (1065). Activation of JAK2 by APOA1 binding to ABCA1 also results in STAT3 binding to ABCA1 and STAT3 activation. JAK2/STAT3 signaling is strongly anti-inflammatory in endothelial cells and macrophages. Recently, STAT3 was shown to induce production of tristetraprolin (TTP), a specific inhibitor of several inflammatory cytokines including TNF-α and IL-1β. TTP acts by binding the 3’ end of the
mRNA of these cytokines and causing destabilization and degradation. Phosphorylation of TTP by p38 causes its inactivation, but APOA1 binding to ABCA1 is also reported to inhibit p38 (2038).

ABCA1 appears to be coupled in some way to Go subunits which activate adenylate cyclase upon APOA1 binding (694). Subsequent PKA activation leads to ABCA1 phosphorylation and increased transport activity. This property was recently utilized to examine efflux of cholesterol to AP0B-deficient serum with or without incubation of free cholesterol-loaded macrophages with a CAMP analog (1038). Active PKA may cause release of cholesterol as microparticles by an unknown mechanism which is greatly facilitated by ABCA1 (455, 1099, 2088). Increasing intracellular CAMP by pharmacological means appears to increase APOA1-mediated cholesterol removal from macrophages and may even increase plasma HDL-C levels (2087).

3. Role of SR-B1 in HDL signaling

Binding of HDL APOA1 to the SR-B1 receptor results in selective uptake of cholesterol ester (that is, without holoparticle degradation) from HDL into hepatocytes and steroidogenic adrenal cells (7). HDL released after interaction with hepatic SR-B1 is thought to be more capable of eliciting efflux from cholesterol-loaded cells. In human macrophages, SR-B1 can mediate net transfer of cholesterol to HDL but apparently in mouse, little if any cholesterol efflux from macrophages occurs via SR-B1 (1523, 1874). Macrophages from human subjects heterozygous for a variant of SR-B1 (P297S) that impaired HDL binding displayed decreased transfer of CE to HDL. In addition, SR-B1-deficient mouse hepatocytes transfected with the abnormal human variant showed impaired uptake of cholesterol from HDL. Finally, increased platelet activation and decreased glucocorticoid production were demonstrated in heterozygous humans, together with a 50% increase in serum HDL-C (to ~70 mg/dl) (1874).

Whole-body SR-B1 KO in mice increased HDL-C and atherosclerosis substantially while liver-specific KO resulted in similar effects on HDL-C but lesser effects on atherosclerosis (1523). However, bone marrow-derived cells (353, 1848) and endothelium deficient in SR-B1 each contribute to excess atherosclerosis without affecting serum lipid levels (1523). Macrophage-specific knockout of SR-B1 had a lesser effect on atherosclerosis, suggesting that HDL interactions with SR-B1 on other cells may be more important (1913, 2081). An altered ratio of plasma unesterified to total cholesterol in SR-B1 deficiency leads to platelet activation and may contribute to the atherosclerosis (772). These studies suggest reverse cholesterol transport is not the only means whereby SR-B1 affects atherogenesis.

Another illustration of anti-atherosclerotic effects realized by HDL without cholesterol transport was recently reported. Vein grafts transplanted to the carotid arteries of ApoE-deficient mice were greatly protected from atherosclerosis when a gel containing HDL was applied to the adventitial side of the transplanted vein. The treatment was strongly anti-inflammatory, promoted recruitment of true endothelial colony forming cells with increased VEGF signaling, and was dependent on the presence of SR-B1 (518).

PDZ domain-containing protein 1 (PDZK1, where PDZ stands for the original recognition of a shared structure in 3 proteins, PSD-95, DlgA, and ZO-1) is an adapter or scaffolding protein which binds to the cytosolic side of SR-B1 and interacts with a number of other membrane-associated proteins to properly locate it in the cell membrane (in some cells) and mediate intracellular signaling. PDZK1 is highly expressed in liver, intestine, and kidney, and in humans, in adrenal, ovary, and testis as well (1523). It is not expressed in macrophages (1752). In hepatocytes, PDZK1 deficiency leads to 95% reduction in SR-B1 expression, but no effect on macrophage SR-B1 expression and little effect in endothelial cells was seen. Nevertheless, PDZK1 is required in endothelial cells for APOA1-induced signaling. Thus, in endothelial cells, APOA1 binding to SR-B1 leads to Src-dependent activation of type 1A PI3K isoforms. Src also seems to mediate activation of Rac and ERK1/2 (1523). PDZK1 is required for Src activation by HDL. Indeed, PDZK1 KO completely blocked HDL-mediated increases in eNOS and promotion of endothelial repair after injury (2113). Increased atherosclerosis was reported for PDZK1 KO in ApoE null mice fed a western diet and led to coronary occlusion when these mice were fed a Paigen-type diet (2034).

The COOH terminus transmembrane segment of SR-B1 contains a conserved glutamine that was recently shown to serve as a membrane cholesterol sensor. In cells expressing normal SR-B1, depletion of cholesterol by either cyclohextrin or HDL caused Src-dependent phosphorylation and activation of eNOS. Converting the conserved glutamine to alanine in the SR-B1 COOH terminus cytoplasmic membrane domain prevented the HDL-induced activation of eNOS. This occurred without affecting the binding of PDZK1 to the SR-B1 COOH terminus. Furthermore, reverse cholesterol transport from HDL to hepatocytes and bile was not affected (1522). The significance for atherosclerosis of this membrane cholesterol sensing function of SR-B1 awaits further studies.

4. HDL and S1P signaling

Several of the atheroprotective effects of HDL on endothelial cells and probably macrophages appear to be mediated not only by apo A-1 but also by lipid components carried in HDL, particularly sphingosine-1-phosphate (S1P) and related lysosphingolipids binding to specific receptors (partic-
ularly S1PR1) (1338, 1888, 1953). HDL carries over 50% of the S1P in plasma (77, 78).

Of potentially great interest is the relatively neglected apolipoprotein, apoM, found on only ~5% of HDL particles (as well as some non-HDL lipoproteins). ApoM apparently mediates virtually all S1P removal from cells and loading onto HDL, as well as subsequent receptor interaction (322, 323). ApoM also seems to be required for normal regeneration of pre-β HDL, possibly by interacting with PLTP or hepatic lipase (HL) (321). Overexpression of apoM was reported to decrease atherosclerosis progression in hyperlipidemic mice (321, 1970), but effects of knockout on atherosclerosis have apparently not been examined (see Table 8).

Production of S1P occurs in response to cytokines that utilize TRAF2 and TRAF6 in their downstream signaling. TRAF2 binds and activates sphingosine kinase 1 (SPHK1). The S1P produced was shown to be a cofactor required for TRAF2 (and probably TRAF6) to function as an E3 ubiquitin ligase (1665). The S1P produced by these reactions then seems to be transported, in part, to HDL by apo M. Thus S1P has the unusual distinction of supporting proinflammatory signaling directly as an intracellular cofactor (and when bound to certain of its GPCRs) while directing anti-inflammatory signaling when presented to S1PR1 by HDL. This duality may help explain the reported strong positive association between CAD and the ratio of non-HDL-bound S1P to HDL-bound S1P (1543).

Binding of APOA1 to SR-B1 not only leads to selective CE uptake in hepatocytes and SR-B1 mediated signaling in endothelial cells but also brings the HDL particle into the proximity of S1PR1 receptors. Injection of mice with an agonist of the S1PR1 receptor or incubation of isolated aorta with S1P ex vivo blocks TNF-induced monocyte adhesion through upregulation of PKB signaling, increased eNOS activity, and possibly inhibition of JNK (172). Activation of the S1PR1 receptor or incubation of isolated aorta with S1P ex vivo blocks TNF-induced monocyte adhesion through upregulation of PKB signaling, increased eNOS activity, and possibly inhibition of JNK (172). Activation of the S1PR1 receptor or incubation of isolated aorta with S1P ex vivo blocks TNF-induced monocyte adhesion through upregulation of PKB signaling, increased eNOS activity, and possibly inhibition of JNK (172).

In VSMC, S1PR1 and S1PR3 seem to promote proliferation and migration (as after acute experimental balloon injury) while S1PR2 is generally inhibitory. Furthermore, the relative proportion of these receptors is modulated during injury repair. Gα12/13 (which may be stimulated by all 5 S1P receptors but primarily in S1PR2–5) is coupled to Rho activation with promotion of vasoconstriction (for VSMC) or disruption of barriers when expressed at high levels (in endothelial cells).

S1PR2 was shown to be highly proatherogenic, particularly in macrophages (see Table 7) (1640). In macrophages, S1PR2 activated cAMP/PKA signaling which inhibits motility and may prevent egress from plaques (S1PR1 may have opposite effects). S1PR2 also promotes TLR4 expression and stimulated macrophage production of IL-1β and IL-18 (1640). In another study, S1PR3 KO led to increased VSMC proliferation and migration (898). Gαi is coupled to S1PR2 and -3 and primarily activates PLCβ and calcium signaling, and hence, vasoconstriction. Thus S1P can promote vasoconstriction or relaxation depending on the balance of S1P receptors and their associated G proteins. In addition, S1P strongly supports angiogenesis through complex signaling and modulation of S1P receptors.

Interestingly, some of the opposing effects of S1P may be controlled by concentration. Thus high levels of S1P (over 1 μM as may occur in the vicinity of aggregated platelets, acting primarily through S1PR3, may stimulate endothelial activation, Weibel-Palade body exocytosis, and adhesion molecule expression. In contrast, low levels of S1P (1–100 nM), acting through S1PR1, may have virtually the opposite effects (1338). In addition to concentration-dependent effects, some of the anti-inflammatory signaling of S1P appears dependent on simultaneous APOA1-mediated stimulation of ABCA1 and SR-B1 (910, 1541).

5. Antioxidant effects of HDL

Spherical HDL can protect endothelial cells and promote NO production by removal of various oxysterols, including...
the spontaneous oxidation product 7-ketocholesterol, by way of the ABCG1 transporter (1763, 1771). HDL also removes 7-ketocholesterol from macrophages and thereby decreases apoptosis in an ABCG1-dependent fashion (1769). Oxidized sterol was found to accumulate when mice were fed a western-type diet. It has been pointed out that AGCG1 may primarily move cholesterol and oxysterols to the inner leaflet of intracellular endosomes and that a variety of molecules other than HDL can serve as the cholesterol acceptor (1762). Nevertheless, HDL helps prevent LDL oxidation or detoxifies oxidized phospholipids in LDL through the HDL-associated enzyme PON1. PON1 KO mice express increased atherosclerosis, while overexpression of PON1, PON3, or the entire human PON1, PON2, PON3 cluster was protective (1607, 1620). These findings contrast with the much more variable outcomes associated with more mild effects on PON1 caused by common human variants (1656). Finally, HDL also binds and inactivates LPS, reducing endotoxin activation through TLR4 (1259).

XI. FURTHER PLAQUE DESTABILIZATION AND PRECIPITATION OF THROMBOSIS

The molecular biology and related signaling of the pathways presented in this section have been the topic of numerous recent reviews. This is particularly true of thrombosis and angiogenesis pathways. For this reason, these topics will be presented in a relatively abbreviated format with a few orienting comments as they relate specifically to atherogenesis.

A. Matrix Metalloproteinases and Cathepsins

Matrix metalloproteinase (MMP) enzymes and cathepsins are generally thought of as powerful extracellular matrix degrading enzymes that cause plaque instability. Virtually all migrating cells must secrete some array of digestive enzymes to move through the extracellular matrix. Nevertheless, MMP functions differ considerably from one to another. A relatively complete series of genetic manipulation studies have now been completed (summarized in TABLE 10). As it turns out, some MMPs (particularly normal levels of MMP-2 and -9) seem to promote plaque stability by promoting VSMC migration and cap formation. In contrast, MMP-12, (and to a lesser extent 13 and 14) and high levels of activated MMP-9 promote inflammation and plaque instability (1295). Tissue inhibitor of metalloproteinases 2 (TIMP2) and TIMP3 inhibited macrophage migration while all the cathepsins tested appeared to support macrophage migration and activation. Thus cathepsin deletion or TIMP2/3 overexpression were protective (see TABLE 10).

B. Thrombosis and Atherosclerosis

Thrombosis followed by thrombus organization clearly plays a role in the progression of advanced, complex plaques, resulting in demonstrable salutary progression of obstructive lesions (233, 1136). However, genetic predisposition to venous thrombosis does not necessarily translate to a predilection to atherosclerosis or a measurable increased risk of MI or other acute coronary events. Arterial thrombi are rich in platelets and poor in fibrin while in venous thromb, platelets are sparse, fibrin predominates, and the clots are friable and prone to large emboli. Well-recognized risk factors for the two conditions are also different. In human studies, genetic factors that clearly increased risk of VTE only slightly increased risk for MI (2032). Similarly, carrying an allele for a bleeding disorder may only slightly decrease risk for MI, if at all (1628, 1667, 1668, 1820). Despite these caveats, a number of new animal models of thrombosis-related genes present promising new avenues for research into this late stage of atherogenesis (see TABLE 10).

At later stages of atherogenesis, ANG II action to increase PAI-I may become important. In ApoE KO mice, the long-acting angiotensin receptor blocker azilsartan decreased PAI-I expression and increased VSMC in plaques, suggestive of greater stability (548). However, whole body PAI-I KO had no effect on atherosclerosis overall (see TABLE 10 under “vaso-vasorum and angiogenesis related”).

Prothrombotic vWF was found to be proatherogenic in mouse models while ADAMTS13 which proteolyzed vWF into small fragments was protective. Tissue factor (TF), the main instigator of clotting, had mixed effects on atherosclerosis, factor V was proatherogenic, while tissue factor pathway inhibitor (TFPI) appeared protective. TFPI blocks the active TF-VIIa complex when activated by active factor X. These and other clotting factors are reviewed in TABLE 10.

Among the most promising late-stage animal models are those that relate to thrombin activation, signaling, and inhibition (183). Thrombin (activated coagulation factor II) has multiple proinflammatory effects on endothelium as well as other cells and stimulates activation of NOX enzymes and ROS production. Thrombin may directly promote VSMC migration and proliferation through protease-activated receptor 1 (PAR1) on VSMC or by increasing PDGF release from endothelial cells (184). Thrombin deficiency was reported to decrease atherosclerosis in animal models (see TABLE 10). Thrombomodulin (TM), expressed on the luminal surface on endothelial cells, binds and inhibits thrombin activation. ApoE KO mice that expressed a defective TM had more than twofold increase in spontaneous atherosclerosis. Carotid cuff placement in these mice greatly increased plaque size, leading to frequent plaque rupture and hemorrhage. Marked reduction in plaque size was reported when mice were treated with the thrombin...
inhibitor dabigatran (181). Similar results were reported with another thrombin inhibitor, melagatran (117, 746). Several other genes that affect clotting or platelet activity are also reviewed in Table 10.

C. Vaso-vasorum

When the thickness of the intima increases beyond just 0.5 mm, hypoxia induces ingrowth of vaso-vasorum (934). In recent years there has been growing appreciation for the potentially major destabilizing effects of these leaky, friable vessels. Postcapillary venules of vaso-vasorum are much more reactive to inflammatory signals than arterial endothelium and were recently shown to harbor 100 times more firmly attached leukocytes than the arterial luminal side (485). Such inflammation is further supported by the lymphoid tissue that can develop in the adventitia, supported by VSMC activation as previously noted. Others have identified adventitial entry through vaso-vasorum as a major source of plaque macrophages (1206). Furthermore, vaso-vasorum invading lipid-rich plaques remains highly permeable due to constant exposure to inflammatory factors. Rather than sudden hemorrhage, vaso-vasorum constantly leaks red blood cells into plaques leading to plentiful red cell markers in unstable plaques despite relatively few recognizable red blood cells. In these large, lipid-rich, inflammed lesions, red cell membranes appear to contribute majorly to free cholesterol which may greatly exacerbate formation of toxic cholesterol crystals in lesional phagocytes. In addition, red blood cells are a source of strongly pro-oxidant heme iron which is thought to favor inflammation and lesion progression when exposed to already inflammed, M1-type macrophages. Such a scenario may ominously favor progression of lesions for some time even after plasma lipoprotein levels are reduced.

Gene manipulation of factors which control the neovascularization of atherosclerotic plaques demonstrate that proliferation of vaso-vasorum substantially aggravates the growth of advanced plaques. Some investigators had hypothesized that treatment of mice to promote angiogenesis (as with G-CSF and GM-CSF) would limit atherosclerosis but were surprised to find the opposite (691). Details of a number of such interventions are presented in Table 10.

Finally, perivascular fat has been suspected to promote increased vascular inflammation from the outside in (1455). In a remarkable series of investigations, transplanting perivascular fat around a carotid vessel was shown to markedly increase intimal proliferation after wire injury, enhance adventitial inflammation, stimulate angiogenesis of new vaso-vasorum, and markedly promote localized atherosclerosis in LDLR KO mice. Furthermore, perivascular fat was much more proinflammatory than fat from other sources and showed greatly increased release of TNF-α, MCP-1 (CCL2), and MIP-1α (CCL3) upon high-fat feeding (1135). These findings provide new impetus for controlling weight gain and other factors that may increase deposition of coronary perivascular fat, recently shown to be strongly associated with atherosclerotic plaques in humans (1114, 1161, 1750).

XII. PERSPECTIVES

As the field of molecular biology and signaling matures and physiologically meaningful pathways emerge, we take satisfaction in recognizing the elegance of many of these systems and seeing opportunities for new interventions. While lengthy, this review is but an introduction, lacking, for example, any but the most cursory mention of negative regulation of many of the presented pathways (which have generally not been the focus of the molecular interventions conducted thus far).

Recently, yet another level of control has been recognized in miRNA (micro RNA). One reviewer likened these 700+ known small transcripts to “conductors of the orchestra” (735). After transcription from nongenic regions and introns, followed by processing in the nucleus by removal of the 5’cap and poly-A tail, pre-miRNA are transported out of the nucleus and further processed, finally ending up in the RNA-induced silencing complex (RISC) where they bind to the 3’ end of many mRNA, leading either to transcriptional repression or complete mRNA degradation. Because a given miRNA can bind many different mRNA molecules, and a particular mRNA can bind multiple different miRNA, the potential for control and its complexity escalates rapidly. More than two dozen miRNA species have been documented to affect vascular function (735). One of these, miR-21, is upregulated in endothelial cells by prolonged laminar flow and silences PTEN expression (1934). This decrease in PTEN due to miR-21 expression resulted in enhanced PKB and eNOS activity and increased NO production. Recently, HDL was shown to be capable of transporting and delivering miRNA, resulting in functional effects, with relevance to atherosclerosis awaiting further study (1877). Furthermore, endothelial cells exposed to laminar flow were shown to release miRNA-containing microvesicles which could penetrate and direct VSMC quiescence as manifest by a more contractile phenotype (752). miR-126, secreted by endothelial cells and found in endothelial-derived microvesicles, suppresses endothelial VCAM-1 expression (723) while disturbed flow increased miR-92a which blocked KLF2 and KLF4 (2006). miR-217 and miR-34a block SIRT1, miR-221/222 block eNOS, miR-27b blocks thrombospondin-1, and so on (2006). Many more findings regarding miRNA are likely forthcoming, but models directly demonstrating an effect on atherosclerosis from miRNA manipulation have apparently not yet been reported.

Despite our incomplete understanding, new innovations in treating and preventing atherosclerotic cardiovascular dis-
ease will likely come from continued creative efforts to understand and intervene in the pathways presented above. That being said, vigorous application of proven interventions to control standard risk factors remains the cornerstone for prevention at this time and finds ample justification in the literature reviewed here (particularly for lipids, blood pressure, and weight control to prevent diabetes). Indeed, experimental atherosclerosis rarely occurs without substantial hyperlipidemia and regression generally occurs with normalization of serum lipids.

The field seems ripe for gleaning new understanding in humans of how dietary fats such as saturated fat (particularly palmitic acid), PUFA, and n-3 fatty acids might affect ceramide signaling, resolution-promoting eicosanoid production, ER stress, and other signaling pathways. Other areas of great interest that may find application in atherosclerosis prevention include potential dietary and pharmacological effects on formation of electrophilic LDL, IKK1/2 inhibition (such as by salicylates or curcumin), manipulation of protective S1P signaling stimulated by HDL, utility of selected electrophilic compounds, and generation of appropriate levels of H2S. The potential benefit of stimulating immune tolerance with increased Treg activity, perhaps using human APOB-100 peptides as in a recent mouse study (751) and/or HSP60, is an important avenue to explore in human trials. The apparent utility of direct thrombin inhibition in advanced atherosclerosis also seems promising. All these potential means of intervention appear to have relatively straightforward application and deserve further study, hopefully with prompt translation into human studies. A human interventional trial to test whether antibody-based inhibition of IL-1β (using canakinumab) can prevent recurrent coronary events is already underway (1485). Other highly directed anti-inflammatory therapies may also move to the forefront in the near future (1054).

In choosing targets for intervention, the importance of balance must always be kept in mind, particularly for long-term use in asymptomatic populations. Reduction of inflammation, such as by increasing Treg (perhaps with vitamin D supplementation), or by targeted inhibition of TNF-α, PKCβ, NOX2, or other interventions must be balanced against risk of immunodeficiency (even if subtle), a judgement that will ultimately require large clinical trials. Direct TNF-α inhibition appears to benefit patients with autoimmune disease, but further understanding of why there is a gradual increase in TNF signaling with age accompanied by a general deficit of cellular antioxidant defenses would perhaps lead to even greater benefits. Pharmacological inhibition of the apoptosis-promoting actions of various signaling molecules (such as p66Shc or JNK) needs to be weighed against a potential increased risk of cancer. Increasing the strongly cytoprotective signaling of PKB paradoxically seems to decrease longevity. Bolstering natural cellular antioxidant defenses seems worth pursuing, recognizing that excessive antioxidant exposure can block the induction of protective antioxidant genes through the Keap1-Nrf2 pathway.

Redundancy, compensatory networks, and the usually mild effects of common variants will make the discovery of new atherosclerosis genes difficult by GWAS or similar statistical approaches in human populations. Nevertheless, inherent limitations are being partly overcome by ever-increasing sample sizes utilized in these studies and application of newer resequencing methodologies. However, the fundamental limitations of human gene discovery have not impeded the explosion in our understanding of molecular pathways mediating atherogenesis nor need they hinder future translation into clinical applications. Indeed, based on the rich store of knowledge already gleaned, that future looks bright indeed.

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DISCLOSURES

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