Cell

Macrophages in the Pathogenesis of Atherosclerosis

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DOI 10.1016/j.cell.2011.04.005

In atherosclerosis, the accumulation of apolipoprotein B-lipoproteins in the matrix beneath the endothelial cell layer of blood vessels leads to the recruitment of monocytes, the cells of the immune system that give rise to macrophages and dendritic cells. Macrophages derived from these recruited monocytes participate in a maladaptive, nonresolving inflammatory response that expands the subendothelial layer due to the accumulation of cells, lipid, and matrix. Some lesions subsequently form a necrotic core, triggering acute thrombotic vascular disease, including myocardial infarction, stroke, and sudden cardiac death. This Review discusses the central roles of macrophages in each of these stages of disease pathogenesis.

Atherosclerosis underlies the leading cause of death in industrialized societies and is likely soon to attain this status worldwide (Lloyd-Jones et al., 2010). Atherosclerosis first involves a decades-long expansion of the arterial intima, a normally small area between the endothelium and the underlying smooth muscle cells of the media, with lipids, cells, and extracellular matrix (Figure 1). Although this process itself rarely leads to major symptoms due to preservation of the arterial lumen, a few of these lesions undergo necrotic breakdown, which precipitates acute, occlusive lumenal thrombosis and its consequences: myocardial infarction (''heart attack''), unstable angina (accelerating chest pain due to ongoing heart muscle ischemia), sudden cardiac death, and stroke (Virmani et al., 2002). This Review discusses the impact of monocyte-derived macrophages in both early atherogenesis and advanced plaque progression.

Lesion Initiation and Early Progression

Atherosclerosis is a focal disease process that occurs predominantly at sites of disturbed laminar flow, notably, arterial branch points and bifurcations. Careful morphological and functional studies of the earliest stages of atherogenesis in human and animal models indicate that the key initiating step is subendothelial accumulation of apolipoprotein B-containing lipoproteins (apoB-LPs; Figure 2) (Williams and Tabas, 1995). ApoB-LPs are made by liver and intestinal cells and consist of a core of neutral lipids, notably cholesteryl fatty acyl esters and triglycerides, surrounded by a monolayer of phospholipid and proteins. Hepatic apoB-LPs are secreted as very low-density lipoproteins (VLDL), which are converted in the circulation to atherogenic low-density lipoprotein (LDL), and intestinal apoB-LPs are secreted as chylomicrons, which are converted by lipolysis into atherogenic particles called remnant lipoproteins.

Monocyte Entry

The key early inflammatory response to retained apoB-LPs, which may be enhanced by oxidative modification of the LPs, is activation of overlying endothelial cells in a manner that leads to recruitment of blood-borne monocytes (Glass and Witztum, 2001; Mestas and Ley, 2008). Activated endothelial cells secrete chemoattractants, or ''chemokines,'' that interact with cognate chemokine receptors on monocytes and promote directional migration (Figure 2 and Movie S1 available online). Importantly, prevention of monocyte entry by blocking chemokines or their receptors prevents or retards atherogenesis in mouse models of atherosclerosis (Mestas and Ley, 2008). It should be noted that, although early apoB-LP retention precedes and triggers endothelial activation and monocyte entry, monocyte-derived macrophages in the lesion may subsequently secrete apoB-LP-binding proteoglycans (Williams and Tabas, 1995). This mechanism likely plays an important role in the amplification of LP retention once lesions become established, which in turn can help to explain why the inflammation in atherosclerotic lesions fails to resolve (Tabas, 2010a).

Monocytes originate from bone marrow-derived progenitor cells, and this early stage of monocyte development may be regulated by cellular cholesterol content in a manner that can affect atherogenesis. Mice whose monocyte progenitor cells have defective cholesterol efflux due to deficiency of ABCA1 and ABCG1 transporters show an increase in circulating monocyte number (monocytosis) and increased atherosclerosis (Yvan-Charvet et al., 2010a). Importantly, both the monocytosis and increased atherosclerosis are reversible by restoring cholesterol efflux from monocytes to high-density lipoprotein (HDL), and there are correlations in humans among high HDL, lower blood monocyte counts, and decreased risk for atherosclerosis (Coller, 2005).

The role of monocyte subsets in atherogenesis has been a topic of great interest throughout the last decade. In mice, hypercholesterolemia is associated with an increase in an inflammatory monocyte subset referred to as Lyc^{hi}, and these cells enter developing atherosclerotic lesions more readily than Lyc^{lo} monocytes. Data from other models of inflammation have

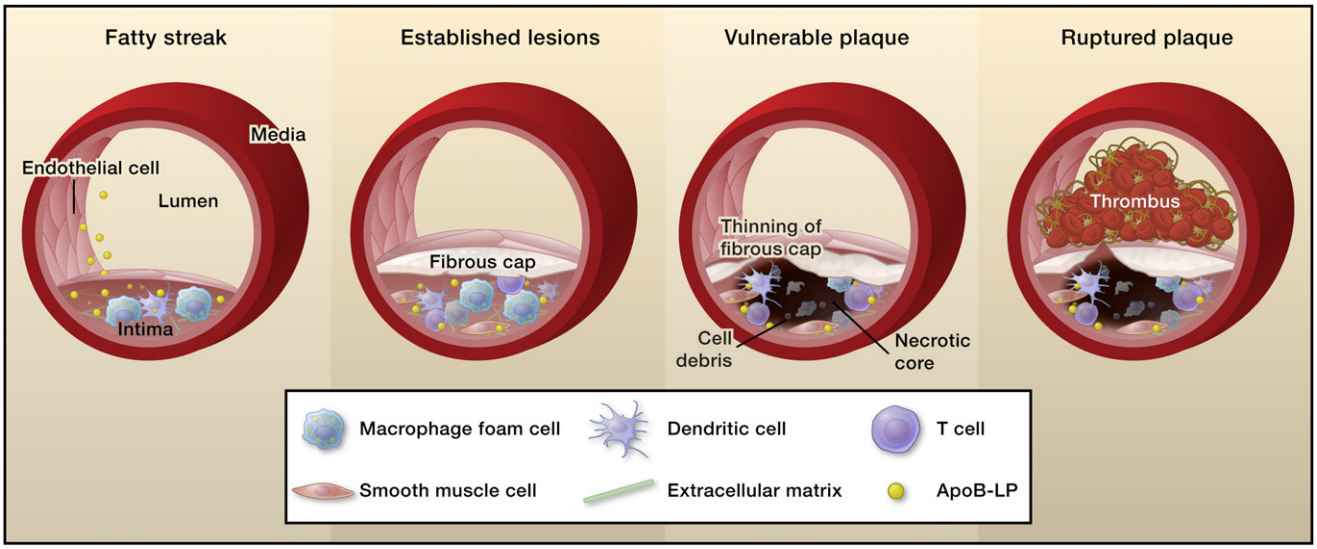

Figure 1. Progression of an Atherosclerotic Lesion

Early fatty streak lesions are characterized by the accumulation of apolipoprotein B-containing lipoproteins (apoB-LPs) in the subendothelial space, which incites the recruitment of dendritic cells and macrophages. As the atherosclerotic lesion progresses, smooth muscle and T cells also infiltrate the intima, and apoB-LP retention is amplified. Vulnerable plaques are characterized by the accumulation of apoptotic cells and defective phagocytic clearance (efferocytosis), resulting in the lipid-filled necrotic core. A thinning fibrous cap decreases lesion stability, making these atherosclerotic plaques susceptible to rupture and the formation of a thrombus.

suggested that Lyc^{hi} monocytes participate in the acute response to inflammation, whereas Lyc^{lo} monocytes are associated with inflammation resolution (Arnold et al., 2007). However, this scenario may not be directly applicable to atherosclerosis because of the chronic nature of the inflammatory process, and maximal suppression of atherogenesis requires genetic manipulations that block entry of both subsets (Tacke et al., 2007). Moreover, there are important differences in the characterization and roles of monocyte subsets in humans versus mice. Thus, the key issue as to whether monocyte subsets—and the macrophages and other cells (e.g., dendritic cells) derived from them—have different roles in atherogenesis or plaque progression remains unresolved.

Following chemokinesis, monocytes become tethered and roll on endothelial cells overlying retained apoB-LPs, notably through the interaction of monocyte P-selectin glycoprotein ligand-1 (PSGL-1) with endothelial selectins (Mestas and Ley, 2008). Monocytes then become firmly adhered to lesional endothelial cells through the interaction of monocyte integrins with endothelial cell ligands, and recent evidence has implicated monocyte type I interferon signaling in this process (Goossens et al., 2010). Immunohistochemical analysis of human lesions and molecular-genetic causation studies in gene-targeted mice suggest that the monocyte integrins VLA-4 (very late antigen-4) and LFA-1 (lymphocyte function-associated antigen 1) and their respective endothelial cell ligands, VCAM-1 (vascular cell adhesion molecule) and ICAM-1 (intercellular adhesion molecule-1), may be particularly important in the setting of early atherogenesis. Moreover, it should be noted that platelet aggregation on endothelium overlying atherosclerotic lesions may also promote monocyte-endothelial interactions by activating NF-κB signaling and expression of adhesion molecules and by depositing platelet-derived chemokines on activated endothelium (Mestas and Ley, 2008; Koenen et al., 2009). Finally, firm adhesion of monocytes is followed by their entry into the subendothelial space (diapedesis) (Kamei and Carman, 2010). Although diapedesis has been studied extensively in other models of inflammation, work in atherogenesis is limited. In particular, molecules that have been implicated in diapedesis in vitro, such as tissue factor and PECAM-1, have not been shown to participate in diapedesis in the setting of atherogenesis per se (Goel et al., 2008; Harry et al., 2008). Part of this complexity may be due to the multiple functions of these molecules and the difficulty of assaying diapedesis in vivo.

Monocyte Differentiation into Macrophages

Driven by macrophage colony-stimulating factor (M-CSF) and probably other differentiation factors, the majority of monocytes in early atherosclerotic lesions become cells with macrophage- and/or dendritic cell-like features (Johnson and Newby, 2009; Paulson et al., 2010). There has been great interest in macrophage heterogeneity in atherosclerotic lesions, particularly regarding macrophages involved in proinflammatory processes (M1) versus those involved in resolution and repair (M2), but a clear picture has not yet emerged from these studies (Johnson and Newby, 2009). Much of the theory in this area has been driven by in vitro studies exploring gene/protein expression patterns and functional attributes of monocytes or macrophages subjected to various treatments, including growth/differentiation factors; cytokines derived from type 1 versus type 2 helper T cells; transcription factors, notably PPARs; and even atherogenic lipoproteins and lipids (Johnson and Newby, 2009; Kadl et al., 2010). However, the situation in the atherosclerotic

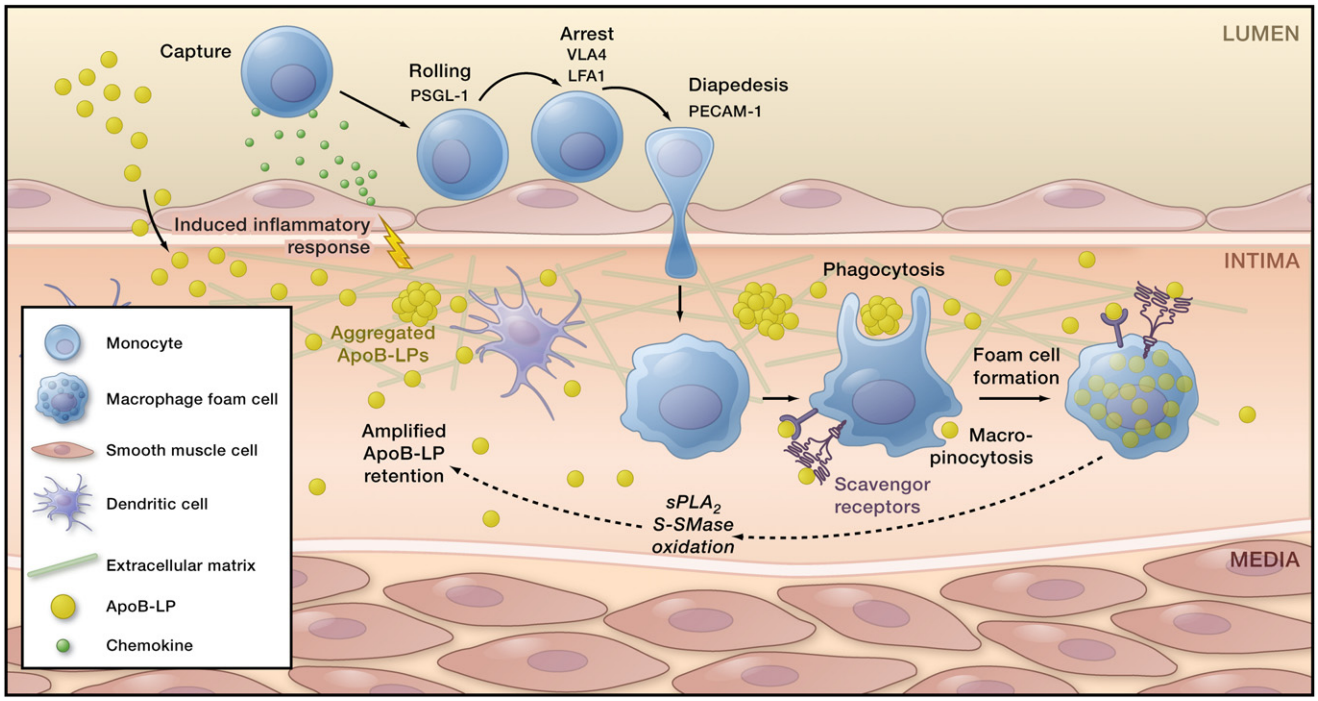

Figure 2. ApoB-LP Retention Promotes Monocyte Recruitment and Subsequent Foam Cell Formation
Apolipoprotein B-containing lipoproteins (apoB-LPs) enter the intima, bind to proteoglycans, and undergo various modifications, including oxidation and hydrolysis by secretory phospholipase A2 ($sPLA_2$) and secretory sphingomyelinase (S-SMase). These modifications incite an inflammatory response characterized by chemokine secretion and altered expression of adhesion molecules by the overlying endothelial cells. The modifications also contribute to lipoprotein aggregation and further promote lipoprotein retention. The inflammatory signals lead to monocyte recruitment into the intima, where they differentiate into macrophages and internalize native and modified lipoproteins, resulting in foam cell formation. Moreover, foam cells can contribute further to, and thus amplify, lipoprotein modifications and retention (dotted arrow).

subendothelium is almost certainly more complex, and there is a significant gap between the in vitro and in vivo observations. Future studies will need to focus on macrophage heterogeneity characterized by differential expression of specific molecules or molecular networks that have *functional* significance for atherogenesis rather than heterogeneity based simply on the aforementioned M1 versus M2 paradigm (Kadl et al., 2010). Moreover, as discussed below, any discussion of "macrophage heterogeneity" must take into account the role of dendritic-like cells and the plasticity of myeloid-derived cells.

Foam Cells

Even at very early stages of atherogenesis, many macrophages and dendritic-like cells have membrane-bound lipid droplets in the cytoplasm. These lipid-loaded cells are called "foam cells," and their formation begins when phagocytes ingest and process apoB-LPs (Figure 2 and Movie S1). The mechanism of this uptake is a widely studied and hotly debated area. Early work suggested that uptake of oxidized LDL occurs via scavenger receptors, notably the type A scavenger receptor (SRA) and a member of the type B family, CD36 (Kunjathoor et al., 2002). However, recent gene-targeting studies in apolipoprotein E (ApoE-deficient mice), a widely used model of atherosclerosis in which plasma lipoproteins are elevated due to absence of the lipoprotein-clearing protein ApoE, indicate that additional mechanisms of foam cell formation are also operational in atherosclerosis (Moore et al., 2005; Manning-Tobin et al., 2009). In vitro work offers plausible mechanisms, including phagocytosis of matrix-retained and aggregated LPs and fluid phase pinocytosis of nonretained native LDL (Tabas et al., 1993; Kruth et al., 2005). Further mechanistic and in vivo studies are needed to fully assess the relative importance of these processes, taking into account stage and location of lesions and the particular model being investigated.

Once ingested, the cholesteryl esters of the LPs are hydrolyzed in late endosomes to cholesterol, often referred to as free cholesterol, and fatty acids (Maxfield and Tabas, 2005). Late endosomal free cholesterol is trafficked to peripheral cellular sites by poorly understood mechanisms involving the late endosomal proteins NPC1 and NPC2 and the lipid lysobisphosphatidic acid. Recent work also points to a possible role of the ABC transporter protein ABCG1 in this process (Tarr and Edwards, 2008). Delivery of free cholesterol to the endoplasmic reticulum (ER) has important roles in downregulating both LDL receptors and endogenous cholesterol synthesis by suppressing the sterol-regulatory element binding pathway (SREBP) (Brown and Goldstein, 1997). Moreover, the free cholesterol undergoes re-esterification to cholesteryl fatty acid esters (the "foam" of foam cells) by the ER enzyme acyl-CoA:cholesterol ester transferase (ACAT) (Brown et al., 1980). Defects in ACAT function or processes involved in cholesterol efflux from cells lead to free cholesterol-induced cytotoxicity and may promote macrophage death in advanced lesions, or "atheromata" (discussed below).

Free cholesterol released from lysosomes and from rehydrolyzed cholesteryl ester droplets can also traffic to the plasma membrane and thus be available for efflux out of the cell (Tall et al., 2008; Rothblat and Phillips, 2010). Cholesterol efflux is thought to be a major process involved in plaque regression when hypercholesterolemia is reversed. This expansive area has been covered in many reviews over the last decade, and here we will briefly summarize several key points. The mechanisms and exact route of cholesterol transport to the plasma membrane are not fully known, although Golgi-to-plasma membrane vesicular transport may be involved (Chang et al., 2006; Maxfield and Tabas, 2005). Interestingly, trafficking of free cholesterol out of lysosomes may become defective in lesional macrophages, which would constitute a barrier to cholesterol efflux and lesion regression (Jerome, 2006). Once at the plasma membrane, cholesterol is transferred to the outer leaflet, where it is removed from the cells by ABCA1- and ABCG1-mediated transport to apolipoprotein A1 and HDL, respectively, or by ''passive diffusion'' to cholesterol-poor HDL (Tall et al., 2008; Rothblat and Phillips, 2010). As predicted, deletion of both ABCA1 and ABCG1 in macrophages enhances atherosclerosis in mice (Tall et al., 2008). Interestingly, deletion of ABCG1 alone in macrophages suppresses atherogenesis, which has been ascribed to either compensatory induction of ABCA1 and ApoE or to ''beneficial'' depletion of early lesional macrophages by oxysterol-induced apoptosis (Ranalletta et al., 2006; Tarling et al., 2010).

Extensive in vitro and in vivo work has focused on how sterol-regulated transcription factors, LXRα and LXRβ (LXR), induces ABCA1 and ABCG1 and, through this and other mechanisms, promote regression of foam cell lesions (Calkin and Tontonoz, 2010). Evidence that ABC transporters, LXR, and other molecules carry out cholesterol efflux and ''reverse cholesterol transport'' in vivo has come from studies in which wild-type and gene-targeted mice have been injected with macrophages loaded with radiolabeled cholesterol, and then the appearance of label in blood and feces is followed (deGoma et al., 2008). As a therapeutic strategy to promote lesion regression, investigators have attempted to enhance macrophage cholesterol efflux by increasing HDL or HDL-like particles or by increasing ABC transporters (Tall et al., 2008; Rothblat and Phillips, 2010). Examples include cholesteryl ester transfer protein inhibitors, LXR activators, and HDL mimetics (see section below on therapeutic strategies). Though no drugs have yet been approved for this purpose, this set of strategies continues to be a major focus in cardiovascular drug discovery.

Macrophage Cholesterol Loading

A central question in atherogenesis is how the loading of macrophages with lipoprotein-derived cholesterol alters macrophage function, particularly with regard to specific proatherogenic processes. Although markedly excessive loading of free cholesterol in the face of defective cholesterol esterification can have profound effects on macrophages in advanced lesions (discussed below), the influence of cholesteryl ester storage is less certain because it is stored in membrane-bound neutral lipid droplets in the cytoplasm and is thus sequestered from cellular membranes (Maxfield and Tabas, 2005). However, even with normal esterification, macrophages exposed to atherogenic lipoproteins may have some degree of enrichment of free cholesterol in the plasma membrane, which may enhance inflammatory signaling through clustering-mediated activation of signaling receptors due to the increased order parameter of cholesterol-enriched membranes (Yvan-Charvet et al., 2007; Zhu et al., 2008; Tang et al., 2009). In addition, other proatherogenic lipids delivered to cells by lipoproteins, particularly modified lipoproteins, may have potent effects. As an example, oxysterols and oxidized phospholipids delivered through the uptake of oxidatively modified forms of lipoproteins can induce macrophage apoptosis (Seimon et al., 2010b).

As a new approach to the question of how lipoprotein loading affects macrophage biology, a recent study used liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) to identify proteins secreted by macrophages using an in vivo model of unloaded versus cholesterol-loaded macrophages (Becker et al., 2010). Gene ontology analysis of proteins differentially expressed by peritoneal macrophages obtained from chow- versus fat-fed *Ldlr*-deficient mice, which have high levels of LDL and develop atherosclerosis when placed on a high-fat diet, revealed networks involved in cytoskeletal regulation, vesicle-mediated transport, and lipid binding. Interestingly, a subset of the appeared to be mediated by a decrease in ApoE expression in the cholesterol-loaded macrophages. In terms of relevance to atherosclerosis, a very high percentage of these proteins have been implicated in lesion development in various gene-targeted mouse models, and when *Ldlr* knockout mice were administered two drugs that decrease atherosclerosis—a statin and a thiazolidinedione—the differences in macrophage protein profiles were markedly decreased. This study not only reveals potentially important information about how macrophages respond to lipoprotein loading in vivo, but also provides an example of how systems biology approaches may be applied to this area of research.

Inflammatory Activation of Lesional Macrophages

The mechanisms and consequences of macrophage-mediated inflammation comprise a central topic in atherosclerosis research (Figure 3). Studies using immunohistochemistry and RNA isolated from lesional macrophages by laser capture microdissection have clearly identified markers of inflammation in macrophages in human and animal plaques (Shibata and Glass, 2009). Moreover, there have been hundreds of studies examining the effect of injected anti- or proinflammatory reagents or of targeting pro- or anti-inflammatory genes in animal models of atherosclerosis. In most cases, manipulations that decrease inflammation have a protective effect on lesion area, and vice versa. However, given the importance of inflammatory pathways in endothelial cells (discussed above), the role of macrophage inflammation per se can only be determined through studies that specifically target this cell type.

Kanters et al. (2003) selectively ablated the *Ikk2* gene, which is involved in NF-κB activation in macrophages (and neutrophils) with surprising results. The resultant mice were used as bone marrow donors to *Ldlr* knockout mice, which were then fed a high-fat ''Western''-type diet for 10 weeks. Although macrophages isolated from the chimeric mice had an ~50% decrease in NF-κB activation and lipopolysaccharide-induced TNFα

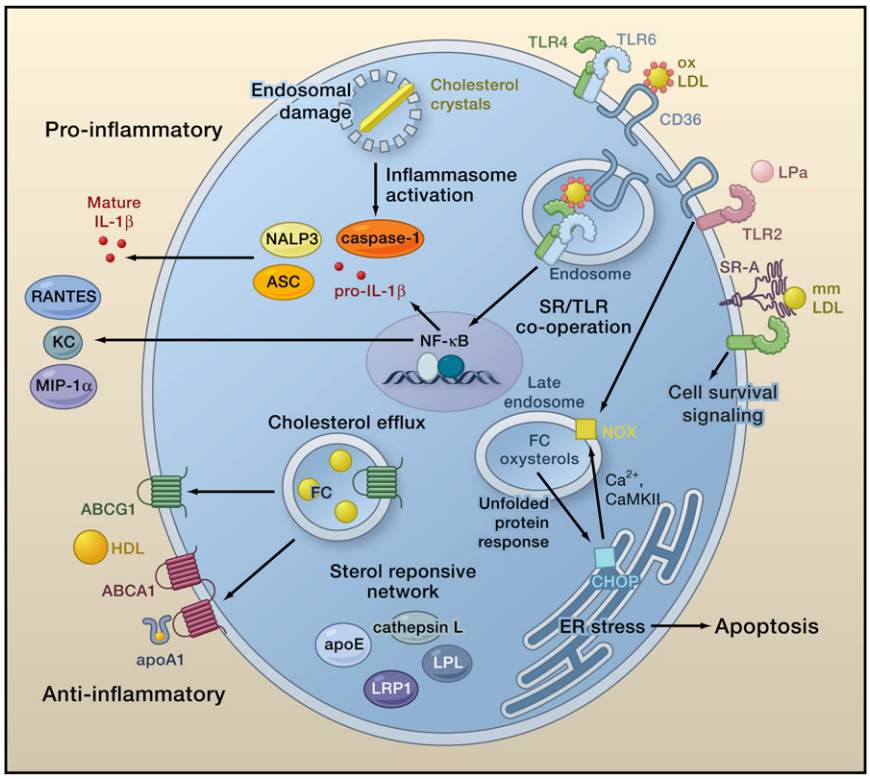

Figure 3. Signaling Pathways in Macrophages of Atherosclerotic Lesions

Various pro- and anti-inflammatory forces act on the macrophage in atherosclerotic lesions, leading to activation of downstream cascades, such as the inflammasome, scavenger receptor (SR)/Toll-like receptor (TLR) cooperative signaling, endoplasmic reticulum (ER) stress, expression of the sterol responsive network, and efflux of cholesterol via ABCA1 and ABCG1 transporters. FC, free cholesterol.

secretion, they showed a surprising increase in lesion area compared with the control group. As an illustration of the importance of cell type specificity in the investigation of atherosclerosis, a study in *Apoe* knockout mice showed that endothelial-targeted inhibition of NF-κB signaling results in a decrease in lesion area, which was associated with a decrease in recruitment of macrophages to lesions (Gareus et al., 2008). Although no definitive mechanism linking increased lesion area to macrophage NF-κB suppression was elucidated in the study by Kanters et al. (2003), LPS-treated monocyte-derived macrophages cultured from these mice showed decreased secretion of interleukin-10 (IL-10), which is athero-protective in mice (Han et al., 2010).

In terms of pathways through which NF-κB becomes activated in lesional macrophages, there are many studies showing that global deletion of various members of the Toll-like receptor (TLR) family or their adaptors decrease lesion area in fat-fed *Ldlr*- or *Apoe*-deficient mice (Figure 3; Curtiss and Tobias, 2008). One of the few studies to address the role of TLR signaling in macrophages in vivo showed that fat-fed *Ldlr*-deficient mice transplanted with bone marrow from *Tlr2* knockout mice had decreased lesion area only when the mice were challenged with a TLR2 activator (Mullick et al., 2005). In addition, a newly described TLR heterodimer of TLR4/TLR6 has been shown to cooperate with CD36 in activating NF-κB in response to oxidized LDL (Stewart et al., 2010). Stimulation of the macrophage CD40 receptor by its ligand, CD40L, may also trigger NF-κB activation, and fat-fed *Ldlr* knockout mice transplanted with bone marrow from *Cd40* knockout mice showed a reduction in lesion area and inflammation (Lutgens et al., 2010).

A fascinating new area is the role of macrophage inflammasome signaling in atherosclerosis (Figure 3). When macrophages are exposed to crystalline material, IL-1β and IL-18 protein maturation and secretion are effected through an inflammasome complex involving multiple proteins, notably NLRP3, ASC, and caspase-1 (Duewell et al., 2010; Rajamäki et al., 2010). This pathway can be induced in cultured macrophages and in mouse peritoneal macrophages in vivo by cholesterol crystals, which have been shown to exist even in early atherosclerotic lesions. Cholesterol crystal-induced macrophage inflammation involves phagolysosomal disruption, inflammasome components, and the lysosomal proteases cathepsin B and cathepsin L (Duewell et al., 2010; Rajamäki et al., 2010). *Ldlr* knockout mice fed a Western diet and transplanted with marrow cells lacking NLRP3 or ASC had a decrease in lesion size and in levels of IL-18 (Duewell et al., 2010).

Macrophages in Advanced Atherosclerosis

As described in the previous sections, atherosclerosis is a nonresolving inflammatory condition, and monocytes continue to enter plaques and differentiate into macrophages as atherosclerotic lesions progress. Importantly, macrophages contribute to size-independent changes in plaque morphology, notably formation of the necrotic core and thinning of the fibrous cap, which characterize the "vulnerable" plaque (Figure 4). Analysis of these processes in advanced plaque progression, e.g., through analysis of intravascular ultrasound data, may be particularly relevant to acute atherothrombotic cardiovascular disease in humans (Stone et al., 2011). Lumenal thrombosis requires communication between procoagulant and prothrombotic factors in the intima with coagulation factors and platelets in the lumen. In ~70% of autopsy specimens that successfully identify the lesion responsible for an acute vascular event (culprit lesion), this breach is manifested as a rupture site (Virmani et al., 2002). The rupture site, which is often located to the side (shoulder) of raised lesions, is almost always in close proximity to areas of plaque necrosis (necrotic core) and is associated with thinning of the protective scar (fibrous cap) that normally covers inflamed atheromata. One of the key questions in the atherosclerosis field is how macrophages contribute to the features of such culprit lesions.

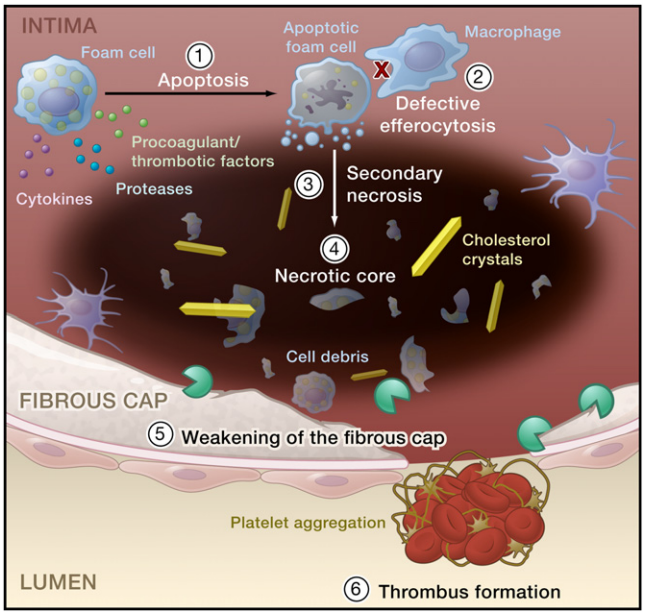

Figure 4. Macrophages in Advanced Atherosclerotic Lesions

(1) Macrophage foam cells undergo apoptosis as a result of prolonged endoplasmic reticulum (ER) stress and other stimuli. (2) These apoptotic cells are not effectively cleared by macrophages (defective efferocytosis) in advanced lesions, (3) leading to secondary cellular necrosis. (4) Secondary necrosis, over time, contributes to the formation of a necrotic core. (5) Smooth muscle cell death and protease degradation of the extracellular matrix weakens the fibrous cap, making it susceptible to rupture. (6) Exposure of the thrombogenic material in the lesion causes platelet aggregation and thrombus formation. Also note that living macrophages in advanced plaques can contribute to vulnerable plaque formation through the secretion of cytokines, proteases, and procoagulant/thrombotic factors.

Fibrous Cap Thinning

Any process that decreases the synthesis of fibrous cap collagen by intimal fibromyoblast-like smooth muscle cells (SMCs) and/or contributes to cap collagen degradation would be expected to promote the formation of plaques prone to rupture (Figure 4). Vulnerable plaques show evidence of SMC death and decreased numbers of SMCs, and in vitro data show that macrophages can trigger apoptosis in SMCs by activating their Fas apoptotic pathway and by secreting proapoptotic TNFα and nitric oxide (Boyle et al., 2003). Macrophages may also decrease collagen synthesis in intimal SMCs without actually killing the cells. For example, in regions of vulnerable plaques that have defective clearance of apoptotic cells (discussed below), macrophage secretion of TGFβ may be decreased (Fadok et al., 1998), which would deprive neighboring SMCs of this important inducer of collagen biosynthesis.

Macrophage-derived matrix metalloproteinases (MMPs) may also be involved in thinning of the fibrous cap (Figure 4). MMPs are a family of protease-activated enzymes that can degrade various types of extracellular matrix (ECM) proteins. The regulation of MMPs by athero-relevant factors is complex and is influenced by transcriptional inducers and repressors and by protease activators and inhibitors. Moreover, once activated, certain MMPs can activate other ones.

Studies showing a temporal and spatial correlation between the presence of macrophages in rupture-prone shoulder regions of plaques, thinning of the fibrous cap in these regions, and local accumulation of activated MMPs, especially MMP-2 and MMP-9, have stimulated great interest in the potential role of MMPs in plaque rupture (Galis et al., 1994). Unfortunately, because mouse models of atherosclerosis do not recapitulate plaque rupture, specific molecular hypotheses in advanced atherosclerosis have been difficult to test in vivo through gene-targeting studies, leading investigations to focus on the endpoints of atherosclerotic lesion area or collagen content or aneurysm development instead. The lesion area studies have yielded results that have been difficult to synthesize into a unified, mechanism-based hypothesis. However, when the endpoint of advanced plaque collagen content has been studied in mouse models lacking global or macrophage-derived MMP-13 and -14, respectively, increases in plaque interstitial collagen were observed (Deguchi et al., 2005; Schneider et al., 2008). In a study that attempted to look directly at plaque disruption, macrophage overexpression of wild-type MMP-9 had little effect in *Apoe*-deficient mice due to a lack of MMP activation in plaques, but overexpression of a constitutively active mutant form of MMP-9 resulted in plaque fissuring (Gough et al., 2006).

In summary, observational data in vulnerable human plaques and the known biology of MMPs have generated plausible hypotheses regarding the role of certain MMPs in fibrous cap thinning and plaque rupture. Moreover, there are other in vitro and in vivo data implicating roles for macrophage-derived serine proteases, such as neutrophil elastase, and cysteine proteases, such as cathepsins S, K, and L, in the degradation of elastin and collagen in advanced lesions (Liu et al., 2004). However, the few molecular causation studies in mice addressing matrix protease hypotheses have had to rely on the endpoints of plaque collagen or elastin content, fibrous cap thinning, and matrix fissuring. New mouse models of true plaque rupture and ideally acute lumenal thrombosis are needed to validate these ideas and to provide the impetus for testing MMP inhibitor strategies in humans.

Plaque Necrosis

A second critical feature of dangerous plaques is the necrotic core (Figure 4), which contributes to inflammation, thrombosis, proteolytic plaque breakdown, and physical stress on the fibrous cap (Virmani et al., 2002). Necrotic cores arise from the combination of apoptosis of advanced lesional macrophages and defective phagocytic clearance (or efferocytosis) of the apoptotic macrophages in advanced plaques (Tabas, 2010a). This combination is critical: macrophage death also occurs in early atherosclerotic lesions, but here, efferocytic clearance is efficient, leading to decreases in lesion cellularity, inflammation, and plaque progression rather than an increase in plaque necrosis.

A number of processes in advanced lesions may trigger macrophage death, and it is almost certain that a combination of factors and processes plays a role in vivo. Examples include growth factor deprivation, oxidative stress, and death receptor activation by ligands that exist in advanced atheromata. A potential role for these and other processes is supported primarily by studies with cultured macrophages, but, in most cases, evidence of relevance in vivo through molecular-genetic causation studies is lacking. In this regard, several studies stand out in

terms of their effects on macrophage-induced plaque necrosis, including the detrimental effect of NF-κB suppression (Kanters et al., 2003) and the beneficial effects of interrupting type I interferon signaling (Goossens et al., 2010) or TLR2/4 signaling (Seimon et al., 2010b).

Another emerging concept that has gained in vivo support is that prolonged activation of endoplasmic reticulum (ER) stress pathways, primarily the unfolded protein response (UPR), contributes to macrophage death and subsequent plaque necrosis in advanced atheromata (Figure 3; Tabas, 2010b). The UPR functions in normal physiology to correct disequilibria in ER function, but when ER stress is abnormally prolonged, persistent expression of the UPR effector C/EBP-homologous protein (CHOP) can trigger apoptosis (Tabas and Ron, 2011). There are many molecules and processes in advanced lesions that can lead to UPR activation, and markers of ER stress are elevated in these lesions. Importantly, CHOP expression and apoptosis show a strong correlation with stage of human coronary artery lesions, with both parameters markedly increased in plaques with vulnerable morphology (Myoishi et al., 2007). Moreover, the deletion of CHOP in mouse models of advanced atherosclerosis suppresses lesional macrophage death and plaque necrosis (Thorp et al., 2009; Tsukano et al., 2010). Several downstream mediators of CHOP have been implicated in this process, including the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), which is a central integrator of the apoptotic execution pathway through activation of Fas, outer mitochondrial membrane permeabilization, STAT1, and NADPH oxidase (NOX) (Timmins et al., 2009). In this context, a recent study implicated oxidative stress and NOX in plaque necrosis in aged *Ldlr*-deficient mice (Collins et al., 2009).

A potent inducer of prolonged ER stress in macrophages is insulin resistance, and advanced plaques of type 2 diabetics are characterized by very large necrotic cores independent of lesion size (Tabas et al., 2010). Macrophages with defective insulin signaling, such as occurs in type 2 diabetes, have an amplified response to ER stress, leading to increased macrophage apoptosis and plaque necrosis in a mouse model of atherosclerosis (Han et al., 2006). Another potential inducer of ER stress in the settings of insulin resistance and obesity are elevated saturated fatty acids (SFAs). These macrophage apoptosis pathways induced by ER stress likely complement other consequences of diabetes on advanced lesional macrophages, such as dyslipidemia-mediated proinflammatory effects, to promote advanced plaque progression.

Two key concepts related to the mechanism and in vivo relevance of ER stress-induced macrophage death are the presence of compensatory cell survival pathways and the role of a "two-hit" apoptotic process. In vitro and in vivo data indicate that there is activation of a number of compensatory cell survival pathways, including those involving NF-κB, Akt, p38α, and autophagy (Tabas, 2010b). Importantly, the onset of apoptosis correlates with eventual suppression of these pathways, and apoptosis can be accelerated by experimentally interfering with the pathways. The two-hit concept is based upon the likelihood that ER stress in vivo may be more subtle than is often modeled in vitro, where one can induce macrophage apoptosis by high concentrations of ER stress-inducing agents. In this context, subapoptotic, prolonged ER stress in macrophages can set the stage for apoptosis induction by other subapoptotic factors, leading to a synergistic apoptotic response. Remarkably, a potent and athero-relevant "second hit" in ER-stressed macrophages is activation of innate immune pattern recognition receptors (PRRs), which may have evolved as a host defense response against invasive organisms that require living macrophages to survive (Seimon et al., 2010a). Two major classes of PRRs, scavenger receptors (SRs) and TLRs, have been implicated in atherosclerosis and are likely to be relevant to apoptosis in ER-stressed macrophages. These PRR pathways trigger apoptosis in ER-stressed macrophages by suppressing compensatory cell survival pathways that are induced by prolonged ER stress and by adding to the burden of oxidative stress. Importantly, atherosclerotic mouse models lacking two SRs, SR-A and CD36, or two TLRs, TLR4 and TLR2, are partially protected against advanced lesional macrophage death and plaque necrosis in a manner that cannot be explained simply by other effects on early plaque progression. In addition, a lipoprotein in humans called lipoprotein(a) [Lp(a)], which is highly associated with atherothrombotic vascular disease and a carrier of oxidized phospholipids (Bergmark et al., 2008), is a potent trigger of apoptosis in ER-stressed macrophages through oxidized phospholipid-mediated activation of CD36/TLR2, a second hit pathway (Seimon et al., 2010b). Notably, a study in a transgenic rabbit model showed that increased plasma Lp(a) led to a marked increase in plaque necrosis without altering signs of early lesion development (Sun et al., 2002).

Defective Efferocytosis

Macrophage apoptosis by itself will not trigger plaque necrosis. Rather, plaque necrosis results when apoptotic macrophages are not sufficiently cleared by phagocytes (efferocytosis) (Tabas, 2010a). Efficient efferocytosis of apoptotic macrophages results in at least three critical protective effects: (1) it clears the cells before membrane damage leads to extracellular leakage of toxic intracellular material; (2) it triggers an IL-10- and TGFβ-mediated anti-inflammatory response in the efferocytes; and (3) it promotes survival of the efferocytes themselves so that they do not succumb to potentially toxic factors in the ingested apoptotic cells. This cell survival effect involves robust esterification and efflux of cholesterol, efflux of proapoptotic oxidized lipids, and activation of Akt and NF-κB cell survival pathways.

Efferocytosis is effected through phagocytic receptors (such as the receptor tyrosine kinase MerTK and low-density lipoprotein receptor-related protein 1 [LRP-1]), apoptotic cell ligands (such as phosphatidylserine and C1q), and bridging molecules (such as Gas6) that enable recognition of apoptotic cells by the phagocytes (Henson et al., 2001). In advanced atherosclerosis, the combination of apoptotic macrophages and tissue necrosis implies that efferocytosis is defective (Figure 4; Henson et al., 2001). Direct support for this concept came from a study that carefully quantified apoptotic cell-phagocyte association in human advanced plaques and control tonsillar tissue (Schrijvers et al., 2005). In tonsils, as expected, almost all of the apoptotic cells were associated with phagocytes, whereas in advanced atheromata, there were many free apoptotic cells. Similar results have been found in mouse models of advanced atherosclerosis, and when efferocytosis is decreased further through gene

targeting of effector molecules like MerTK, LRP-1, transglutaminase-2, C1q, and MFG-E8, there is increased presence of uningested apoptotic cells, inflammation, and plaque necrosis (Tabas, 2010a). Normal functioning of MerTK and LRP-1 may be particularly important in preventing plaque necrosis because, in addition to their roles in efferocytosis, MerTK suppresses macrophage inflammation, and LRP-1 promotes macrophage survival (Yancey et al., 2010).

The mechanisms of defective efferocytosis in advanced plaques are not known. Overwhelming apoptosis is not likely to be a major factor because efferocytosis is a high-capacity process (Henson et al., 2001), and when apoptosis is increased through genetic manipulations in early atherosclerotic lesions, in which efferocytosis is not defective, apoptotic cells are efficiently cleared (Tabas, 2010a). Rather, it is likely that efferocytosis itself becomes defective in advanced lesions. Several possible mechanisms might contribute to defective efferocytosis, including oxidative stress-induced efferocyte death resulting from defective cholesterol efflux after apoptotic cell engulfment (Yvan-Charvet et al., 2010b); LP-associated hydrolysis of oxidized phosphatidylserine on the surface of apoptotic cells by phospholipase A2 (PLA_2), which is a ligand for efferocytes (Wilensky and Macphee, 2009); and protease-mediated cleavage of the efferocytosis receptor MerTK (Sather et al., 2007). Another mechanism that may be relevant to obesity is based on in vitro and in vivo studies showing that macrophages exposed to saturated fatty acids have defective engulfment of apoptotic cells, perhaps due to changes in plasma membrane structure (Li et al., 2009). These and other ideas will require careful assessment in vulnerable plaques and then genetic causation testing in mouse models of advanced atherosclerosis.

Dendritic Cells and Atherosclerosis

The distinction between macrophages and dendritic cells (DCs) is a controversial topic, and it is further compounded by the likelihood of plasticity between these two types of cells (León et al., 2005). For the purpose of this section, DCs will be defined as immune cells that internalize, process, and present antigen, leading to activation or suppression of T cells. Both pre-atherosclerotic susceptible regions of arteries and established atheromata are populated with cells that have DC-like properties (Paulson et al., 2010; Choi et al., 2009). The exact origins of arterial wall and lesional DCs have not yet been definitively delineated. A study in fat-fed *Apoe* knockout mice suggests that a population of DCs in atherosclerotic lesions originates from Ly-$6C^{lo}$ monocytes (Tacke et al., 2007). In terms of functional significance, there is some evidence that lesional DCs present antigen to and activate lesional T cells (Bobryshev, 2005). Moreover, early lesional DCs show two fundamental characteristics that were previously ascribed to early lesional macrophages: proliferation and foam cell formation (Paulson et al., 2010). Attempts to probe these events through in vivo causation studies have yielded mixed results. For example, whereas acute ablation of DCs in *Ldlr*-deficient mice fed a high-fat diet for 5 days resulted in a 55% decrease in foam cell lesion area (Paulson et al., 2010), lesion area was only modestly decreased in 10-week fat-fed *Gmcsf* and *Ldlr* double-knockout mice, which is a model of chronic DC deficiency (Shaposhnik et al., 2007). Finally, in the setting of rapid and robust plasma cholesterol unloading in a mouse model of atherosclerosis, monocytic cells expressing CD11c, an often used DC marker, emigrated from lesions in a CCR7-dependent manner, leading to plaque regression (Feig et al., 2010). These intriguing studies warrant further study of the role of DCs in atherosclerosis.

Methods of Investigation and Their Limitations

The reliability of the data cited in this Review is only as good as the methods used to generate and analyze the data. Thus, it is important to understand the limitations of both mechanistic studies using cultured macrophages and in vivo studies using animal models. There are many different types of cultured macrophage models, with important differences between them and actual lesional macrophages. In general, studies with macrophages differentiated and activated in vivo, such as those isolated from the peritoneum of mice treated under various conditions, are probably the most relevant to atherosclerosis, particularly in the setting of hypercholesterolemia, which promotes loading with lipoprotein-derived lipid (Becker et al., 2010). On the other hand, the use of immortal and highly proliferative macrophage cell lines, many of which are derived from cancer cells, may yield results that have less relevance to lesional macrophages. However, the only way to determine the physiologic significance of findings with any cultured macrophage model is to test the findings in animal models of atherosclerosis and to show links with human studies.

In that context, there are several important points to consider with regard to animal studies that attempt to explore macrophage-based hypotheses of atherosclerosis. In an attempt to improve specificity for macrophages, many studies use bone marrow transplantation of irradiated mice with donor cells derived from bone marrow or fetal liver. However, such studies may suffer from the effects of aortic irradiation and from the fact that all bone marrow-derived cells are affected, including neutrophils, T cells, B cells, and mast cells (Schiller et al., 2001). Approaches to improve macrophage specificity in this model include viral transduction of the donor cells with macrophage promoter-driven genes (Gough et al., 2006) and the use of various cre-lox models for cell type-specific knockout, as in Kanters et al. (2003). Although the latter approach has proven to be very useful, it is occasionally hampered by heterogeneity in cre-mediated recombination among individual lesional macrophages, and LysM promoter driving Cre expression also targets neutrophils, which are becoming increasingly recognized as participants in atherosclerosis (Baetta and Corsini, 2009).

In all cases, the greatest limitation is that animal models of atherosclerosis may only partially reflect processes in human atherosclerosis. As is apparent from the previous sections, most atherosclerosis studies are carried out in mice, and the two most widely used models are high-fat Western-type diet-fed *Ldlr* knockout mice and chow-fed or Western diet-fed *Apoe* knockout mice (Breslow, 1996). *Ldlr*-deficient mice are a model of the rare human disease, homozygous familial hypercholesterolemia, although LDL-induced atherosclerosis is, in general, a very common process in humans. *Apoe*-deficient mice have a form of remnant lipoproteinemia that is rare in humans, but postprandial remnant lipoproteins likely contribute

to atherosclerosis in most humans. Influences of other systemic risk factors in these mice, such as levels of plasma HDL and diet-induced insulin resistance, may or may not mimic certain features of humans at risk for atherosclerotic vascular disease. Moreover, most studies use mice on the atherosclerosis-susceptible C57BL6/J background, which has a number of features that likely affect macrophage biology and atherosclerosis, including a proatherogenic increase in the Th1/Th2 balance (Schulte et al., 2008). Of note, the number of mice used in many atherosclerosis studies is far lower than that required by power calculations of appropriate sample size. Moreover, studies in which pure backgrounds are not used may be confounded by major differences in atherosclerosis susceptibility among different strains of mice (Smith et al., 2001), which is not always solved by using littermate controls due to the influence of ''passenger'' genes clustered near the targeted locus (Lusis et al., 2007). The use of pure C57BL6/J ES cells for gene targeting studies, though not as efficient as using other types of ES cells, may be the only way to avoid this problem.

Finally, the choice of atherosclerosis endpoints may influence the usefulness of the data obtained from animal studies. Most studies use cross-sectional lesional area at the aortic root and/or lipid staining of the lumenal surface of the whole aorta (''*en face*'' analysis). These endpoints are relevant for early atherogenesis and for processes related to early to midstage lesion progression. Whereas the pathological features of early to midstage lesions in mice share many common properties with those in humans, lesion area is a poor predictor of clinical disease in humans (Virmani et al., 2002). There has been increasing interest in measuring certain advanced plaque features in mouse models of atherosclerosis, including necrotic core formation, thinning of the fibrous cap, breaches in the subendothelial matrix, and measures of intimal cell apoptosis and clearance of apoptotic cells by lesional phagocytes in situ (efferocytosis). Although these features mimic those in human advanced plaques, mouse atherosclerosis is not a good model of true plaque rupture or acute lumenal thrombosis (Rosenfeld et al., 2002). There are claims that close inspection of the innominate (brachiocephalic) artery in hypercholesterolemic mice or the use of wire-induced injury in various animal models can partially overcome this problem, but true plaque rupture and acute lumenal thrombosis are rarely seen, thus necessitating the development of new models of these processes.

The litany of problems with methods of investigation in this area of research may lead one to be skeptical about studies in the atherosclerosis field. Indeed, the literature on cultured macrophages and gene targeting studies in this area is overwhelming, with many genes being reported to have ''major'' effects, all in the face of a significant number of studies showing opposite results in different laboratories. Moreover, many studies elucidate a mechanism in vitro and show an overall effect on lesion area in vivo but fail to link the two, that is, by demonstrating that the mechanism gleaned from in vitro experiments is operational and causative for the atherosclerosis changes in vivo. However, technical and conceptual advances on many fronts have recently increased the quality of work in this area, and consistent results are apparent in the most carefully conducted and comprehensive investigations. In the end, the true test of validity will be the demonstration that the results are consistent with the results of human genetic studies and with the outcomes of mechanism-based clinical trials.

Therapeutic Strategies

General Principles

The most effective and direct way to prevent or treat atherosclerosis is to decrease subendothelial apoB-LP retention by lowering apoB-LPs in the blood through lifestyle changes and drugs (Steinberg et al., 2008). Studies have shown that apoB-LP lowering both decreases monocyte entry (Potteaux et al., 2011) and promotes macrophage egress from lesions (Feig et al., 2010). Indeed, if circulating apoB-LPs could be lowered below the threshold level required for subendothelial lipoprotein retention prior to lesion initiation, which in Western society occurs in the early teens, atherosclerosis could be completely prevented. However, until this goal can be achieved safely and universally, atherosclerotic disease will remain a major cause of death, particularly in view of the increased global incidence of important risk factors, such as obesity, physical inactivity, insulin resistance, and, in emerging countries, smoking. Thus, translational researchers in this area have considered the concept that therapy directed at the arterial wall in general and macrophages in particular could be additive or synergistic with realistic goals of apoB-LP lowering.

Studies in mice have shown that prevention of monocyte entry into lesions by antibody or gene-targeted neutralization of chemokines, chemokine receptors, or adhesion molecules lessens atherosclerosis (Mestas and Ley, 2008). However, by the time most subjects come under clinical care, many lesions are already far advanced, so simply blocking monocyte entry at this stage may not be able to reverse key aspects of plaque progression, such as necrotic core formation. As mentioned above, reduction of early lesional macrophages through forced apoptosis and subsequent efferocytosis lessens lesion progression (Liu et al., 2005; Arai et al., 2005; Babaev et al., 2008); however, attempts to increase macrophage apoptosis in coexisting advanced lesions that have defective efferocytosis may cause the untoward effect of plaque necrosis and lead to lesion vulnerability. Finally, the macrophage-related mechanisms involved in atherosclerosis and host defense are not easily distinguishable, so many therapeutic approaches along these lines will likely result in increased susceptibility to infectious organisms. These problems could possibly be lessened by local delivery systems, such as atherosclerosis-directed nanoparticles (Chan et al., 2010), encapsulated macrophage-targeted small interfering RNA (Aouadi et al., 2009), and stent-based delivery systems (Nakano et al., 2007) or by identifying and then targeting mechanisms that are more operational in atherosclerosis than in host defense.

Therapeutically inducing cholesterol efflux in macrophages or macrophage precursors by raising HDL and/or cholesterol efflux transporters is likely to have several beneficial, athero-specific effects, including decreasing inflammation related to monocytosis, suppressing activation of inflammatory signaling pathways in macrophages, and increasing efferocytosis by preventing

cholesterol- and oxysterol-induced efferocyte death (above) (Tall et al., 2008). Moreover, HDL may have benefits beyond those related to macrophage cholesterol efflux, including prevention of subendothelial apoB-LP retention, decreasing activation of endothelial cells, and reducing LDL oxidation. Animal studies in which HDL-like particles are administered or in which HDL is raised through genetic techniques show beneficial effects on atherosclerosis. In humans, clinical trials are ongoing to assess the effect of cholesteryl ester transfer protein (CETP) inhibitors, which raise HDL and, importantly, also lower LDL. Although an initial trial in this area showed disappointing results, possibly due to off-target effects, the early results of a second CETP inhibitor trial appear promising (Cannon et al., 2010). Similarly, boosting macrophage ABC transporters through LXR agonists is also beneficial in animal models, and LXR agonists are being developed for trials in humans (Tall et al., 2008) (http://www.bms.com/research/pipeline/Pages/default.aspx). LXR agonists also have anti-inflammatory effects, and they increase the expression of both CCR7, which promotes macrophage egress from lesions, and MerTK, which mediates efferocytosis (Feig et al., 2010; A-Gonzalez et al., 2009). PPAR agonists, like LXR agonists, have potentially beneficial effects on cholesterol metabolism and inflammation in macrophages in vitro and in atherosclerosis in animal models (Rigamonti et al., 2008). However, their actions are complex, multiple cell types are affected, and currently available drugs have off-target effects, some of which may actually exacerbate coronary artery disease.

Antioxidant therapy has been considered as a way to prevent atherosclerosis, in part through effects on macrophage processes (Witztum and Steinberg, 2001). The hope was that antioxidants would prevent foam cell formation and subsequent inflammatory changes by suppressing the formation of oxidized lipoproteins and lipids and by decreasing oxidative stress (Witztum and Steinberg, 2001). To the extent that scavenger receptors mediate oxidized lipoprotein-induced foam cell formation, the aforementioned studies in mice lacking these receptors (Moore et al., 2005; Manning-Tobin et al., 2009) have raised uncertainties about this particular mechanism. Moreover, the results of vitamin E trials in human heart disease have been disappointing (Williams and Fisher, 2005). Nonetheless, this overall strategy should not necessarily be abandoned because oxidized lipoproteins and lipids exist in human atheromata; there are sufficient in vitro mechanistic studies and preclinical studies to support aspects of the oxidative stress hypothesis, including roles in endothelial activation and advanced plaque progression; and it is likely that vitamin E was a poor choice to test this concept in humans. Thus, as we gain more knowledge about specific oxidative processes that contribute to atherosclerosis, such as possible roles for members of the NADPH oxidase family, new therapeutic opportunities in this area may become evident.

Targeting Macrophages in Advanced Lesions

Because only 2%–3% of atherosclerotic lesions cause acute cardiovascular syndromes (Virmani et al., 2002), an efficient strategy may be to therapeutically alter those macrophage processes that are involved in advanced plaque progression. As mentioned above, HDL/sterol efflux-based therapy and well-conceived antioxidant approaches may be relevant to this topic. More direct attempts to block advanced plaque macrophage inflammation may be too nonspecific or subject to adverse effects. For example, blocking TLRs would likely suppress host defense mechanisms, and blocking NF-κB may inhibit an important cell survival pathway in macrophages. A targeted approach that has been considered is inhibition of MMPs by preventing their secretion or activation or by blocking their activity. However, as noted above, this is a complex area with poor understanding of mechanism and lack of definitive causation data in gene-targeting studies. Moreover, the interpretations of preclinical MMP inhibitor drug studies are hindered by the possibility of off-target effects, lack of specificity with regard to members of the MMP family, and lack of documentation of inhibition of MMP activity in plaques.

Our increasing understanding of how macrophages die in advanced lesions has provided new targets that could be the basis for drugs that prevent plaque necrosis, particularly inhibitors of proapoptotic ER stress pathways (Tabas, 2010b). However, a concern is that inhibition of macrophage apoptosis would increase the number of living macrophages in advanced plaques, which might cause damage even in the face of decreased necrotic core formation. A more effective strategy may be to enhance the phagocytic clearance of the dead macrophages, which would result not only in decreased necrotic core formation, but also in fewer macrophages in advanced lesions (Tabas, 2010a). Strategies being explored include proinflammation resolution mediators such as lipoxins, resolvins, and interleukin-10 receptor activators (Serhan et al., 2008); MerTK inhibitors of cleavage (Sather et al., 2007); LXR activators, which increase expression of MerTK, secretion of the efferocytosis effector apolipoprotein E, and ABCA1/G1-mediated efflux of toxic sterols (A-Gonzalez et al., 2009; Yancey et al., 2010; Yvan-Charvet et al., 2010b); and PPARδ activators, which enhance efferocytosis in vitro (Mukundan et al., 2009). As with early inflammatory processes, those involved in advanced plaque progression may require local delivery systems.

Lessons from Human Genetic Studies

The above discussion has considered a relatively large number of potential macrophage targets for therapeutic development in the area of atherothrombosis. To what extent might the results of human genetic studies on atherothrombotic coronary artery disease guide the choice of which targets to pursue? There are several caveats that need to be considered in this area: the genes identified thus far each account for only a small percentage of risk, as expected for a complex disorder; most findings to date are associated with systemic risk factors, such as high LDL or low HDL; and for many genome association findings, the actual gene responsible for the effect is not known. More specifically with regard to this Review, no genes associated with human coronary artery disease have been directly tied to macrophage biology per se. However, several studies have shown that polymorphisms associated with increased levels of Lp(a), an inducer of cell death in ER-stressed macrophages, are associated with risk of coronary artery disease, which is consistent with many clinical studies linking high levels of Lp(a) with acute cardiovascular events (Trégouët et al., 2009). Another risk locus is situated near the gene encoding CXCL12 (SDF-1), which is expressed in atherosclerotic lesional cells,

including macrophages (Abi-Younes et al., 2000). Although it is unclear whether the *CXCL12* gene is affected and, if so, whether CXCL12 is protective or detrimental, recent work has shown that risk of coronary artery disease is associated with higher levels of plasma CXCL12 (Mehta et al., 2011). The locus with the strongest and most consistent association with coronary and peripheral atherosclerosis, but not necessarily acute thrombotic vascular events, is located on chromosome 9p21.3 (Lotta, 2010). This locus is associated with aneurysms in addition to coronary artery disease, potentially indicating a mechanism related to arterial wall remodeling, but the mechanism is not known. The locus contains an antisense noncoding RNA in the *INK4* locus (*ANRIL*) as well as the cell-cycle genes *CDKN2B*, which encodes $p15^{INK4B}$; *CDKN2A*, which encodes $p14^{ARF}$ and $p16^{INK4A}$; and *MTAP*. Several studies have begun to study this genomic region in depth, but much more work is needed to elucidate the mechanism of its association with atherosclerosis and whether it might be associated with macrophage biology.

The Need for Improved Imaging Techniques

How would one test the effectiveness of an anti-atherosclerosis drug targeted at lesional macrophages in humans? Unlike the situation with LDL lowering therapy, there are no easily obtainable endpoints. The only sure sign of effectiveness would be clinical trials showing protection against acute atherothrombotic vascular events, but such trials are extremely expensive, owing to the large number of patients required and the relatively long duration of the observation period. Thus, the drug industry has taken a guarded approach toward the overall concept of arterial-based strategies (Fryburg and Vassileva, 2011). The eventual answer may lie in defining intermediate endpoints that are proven to be reliable for predicting the ability of a drug to decrease acute vascular events. Though circulating biomarkers would be ideal for this purpose, their track record for this type of endeavor has not yet been encouraging. However, given the unique morphology of the plaques that actually cause acute coronary syndromes, imaging modalities focused in this area may offer the best hope. Indeed, this is a highly active area, and new advances in fluorodeoxyglucose-based PET-CT (Positron emission tomography-computed tomography) to image activated lesional macrophages, together with novel high-resolution MRI (magnetic resonance imaging) techniques to assess plaque necrosis, are examples of advances in this area (Sadeghi et al., 2010). Functional imaging based, for example, on detecting MMP activation in lesions is another area in development at the preclinical level (Deguchi et al., 2006). In all likelihood, success will depend on a combination of approaches. Moreover, there will be a significant lag period before such methods can be used routinely to evaluate new drugs because the first step will be to demonstrate that they can accurately predict acute vascular events, followed by repeated demonstrations that changes in these imaging endpoints with athero-protective drugs are directly associated with improvement in clinical outcome.

Conclusions and Perspectives

There are many thousands of papers in the literature on the cell-based mechanisms and in vivo processes operative in atherosclerosis, which is appropriate for the leading cause of death in the industrialized world. The emphasis in this Review has been to highlight major concepts of how macrophages affect plaque initiation and progression. In this regard, it is instructive to compare this current state of knowledge with that reported in a classic and highly cited Review article by Russell Ross in 1986 (Ross, 1986). As expected, progress during the last 25 years has been built upon earlier findings, as illustrated by the appreciation in 1986 that monocyte-endothelial interactions give rise to macrophage-derived foam cells in lesions, which "may represent a form of inflammatory response." However, most emphasis was placed on endothelial "injury" and growth factor-induced smooth muscle cell proliferation rather than the critical role of subendothelial apoB-LP retention in triggering lesion initiation and the nature of the maladaptive inflammatory response that promotes plaque necrosis. The last 25 years have witnessed major advances in understanding the molecular-cellular mechanisms and in vivo relevance of processes involved in plaque development and advanced plaque progression, such as macrophage inflammatory and apoptotic signaling pathways, as well as in monocyte biology and macrophage cholesterol efflux.

There are several principles reviewed herein that are worth reiterating. First, atherosclerosis, including the roles of macrophages in the disease process, would not be the critical topic it is today if we had safe and effective ways of markedly lowering apoB-LPs in every human before they reach their teen years, the time at which lesion initiation begins. Unfortunately, we are far from this goal. Second, most if not all of the processes by which macrophages promote atherothrombotic vascular disease have evolved to optimize our chances of survival to reproductive age. Thus, for example, the inflammatory and oxidative processes through which macrophages promote atherosclerosis are used by these cells to kill infectious organisms (Seimon et al., 2010a), and ER stress-signaling pathways active in advanced plaque progression are essential for macrophage cytokine production during host defense (Martinon et al., 2010). Third, despite the intense effort to determine how macrophages affect atherosclerosis, there is a remarkable number of fundamental issues that remain mysterious. For example, we are far from understanding how the most obvious and distinguishing feature of lesional macrophages, lipid loading, precisely affects macrophage biology and atherosclerotic processes. Solving these mysteries will require a combination of innovative ideas and new and improved methods of investigation. Finally, investigators in the field must continually evaluate how work in this area can be translated into new and useful therapeutic strategies. Even as we continue to attack the unsolved mysteries in this area, there are already an abundance of plausible targets. However, there are two rate-limiting steps: specificity issues, particularly with regard to the possibility of compromising host defense, and the feasibility of evaluating the effectiveness of arterial wall-based therapy in humans without the requirement for long and costly clinical trials that rely on delayed disease endpoints. The former area is likely to benefit from innovative drug delivery systems, such as arterial wall-targeted nanoparticles, and the latter from new imaging procedures focused on the features of the vulnerable plaque. As is the case with the identification of the targets themselves,

both of these challenges in drug development will benefit from continued mechanistic and in vivo studies on the cellular biology of macrophages in atherosclerosis.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one movie and can be found with this article online at doi:10.1016/j.cell.2011.04.005.

ACKNOWLEDGMENTS

This work was supported by NIH grants P01-HL054591, R01-HL075662, R01-HL057560, R01-AG20255, and P01-HL087123. The authors acknowledge invaluable discussions with members of their laboratories and colleagues in the field.

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