

THE *UMUSUAL SUSPECTS*: AN OVERVIEW OF THE MINOR LEUKOCTYE POPULATIONS IN ATHEROSCLEROSIS

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Abbreviations: BCR, B-cell receptor; α -GalCer, α -galactosylceramide; IFN γ , interferon γ ; IL, interleukin; LT, lymphotoxin; MCP-1, monocyte chemoattractant protein-1; MHC, major histocompatibility complex; NK cell, natural killer cell; NKT cell, natural killer T-cell; oxLDL, oxidized low density lipoprotein; PAF, platelet activating factor; PAF-AH, platelet activating factor acetylhydrolase; RAG, recombination activating gene; TCR, T-cell receptor; TGF, transforming growth factor; Th, T helper; TNF, tumor necrosis factor; T_{reg}, regulatory T-cells

Abstract

Atherosclerosis is a complex inflammatory disease process involving an array of cell types and interactions. Although macrophage foam cells and vascular smooth muscle cells comprise the bulk of the atherosclerotic lesion, other cell types have been implicated in this disease process as well. These cellular components of both innate and adaptive immunity are involved in modulating the response of macrophage foam cells and vascular smooth muscle cells to the retained and modified lipids in the vessel wall, as well as driving the vascular chronic inflammation that characterizes this disease. In this review, the involvement of a number of less prominent leukocyte populations in the pathogenesis of atherosclerosis will be discussed. More specifically, the roles of natural killer cells, mast cells, neutrophils, dendritic cells, $\gamma\delta$ T-cells, natural killer T-cells, regulatory T-cells, and B-cells will be addressed.

Supplemental key words:

atherosclerosis, innate and adaptive immunity, natural killer cells, mast cells, neutrophils, dendritic cells, $\gamma\delta$ T-cells, natural killer T-cells, regulatory T-cells, B-cells

Atherosclerosis is a complex and chronic inflammatory disease process affecting large and medium sized arteries. This disease is characterized by the retention and modification of lipids in the vascular wall followed by the subsequent infiltration of inflammatory cells (1). The macrophage foam cell is the predominant inflammatory cell present in the atherosclerotic plaque, and is essential for the development of atherosclerosis (2, 3). As the lesion progresses, the migration of fibro-proliferative vascular smooth muscle cells derived from either the underlying medial layer or alternatively from circulating progenitor cells contribute to the formation of the stabilizing fibrous cap. Overlying the plaque is a layer of endothelial cells, influenced by the local hemodynamic profile and responsible for homing inflammatory cells to this site of retained and modified lipids (4). Finally, T- and B-lymphocytes have been implicated in atherogenesis primarily through cytokine secretion and immunoglobulin production respectively. Over the past 10 years, it has been increasingly recognized that although not required for atherogenesis, T- and B-cells are able to modulate the progression of this disease despite their relatively low numbers in the plaque (5-10). Numerous studies have demonstrated that T-cells in particular have the capacity to modulate the development of atherosclerosis, and their influence is linked to the pro-inflammatory Th1 cytokines or anti-inflammatory Th2 cytokines they secrete (11-14). Although these aforementioned cell types constitute the major cellular players in the current model of atherosclerosis, it has become increasingly recognized that other cellular populations of the innate and adaptive immune system can impact the disease as well (15). In this review we will investigate the involvement of these less prominent leukocyte populations in atherosclerosis, with the hopes of clarifying the role that some of these “unusual suspects” may play in the pathogenesis of this disease.

Cells of the Innate Immune System

Natural Killer Lymphocytes

The natural killer (NK) cell is a bone-marrow derived lymphocyte aptly named for its intrinsic ability to lyse certain tumor cells (16). NK cells are distinct from both T- and B-lymphocytes, and develop normally in immunocompromised mouse models, such as recombination activating gene (RAG) deficient mice (17, 18), indicating that gene rearrangement is not required for NK cell development. NK cells are an important part of the innate immune system. Their primary physiological role is thought to lie in their ability to provide early defense against pathogens during the initial response period while the adaptive immune response is being activated, as well as in viral surveillance and tumor rejection (19). Functionally, these cells act as effectors either through cell-mediated cytotoxicity upon the release of dense cytoplasmic granules containing perforin and granzymes, or through cytokine production, especially interferon γ (IFN γ), thereby activating other effector cells. Cell lysis depends upon perforin forming pores in the cell membrane of target cells through which granzymes, serine proteases, enter the cells and initiate cell death. Additionally, NK cells themselves can become activated through cytokine stimulation. Therefore, NK cells are an important component of the innate immune system through target cell killing and cytokine production, and have been thought to play a role in the developing atherosclerotic plaque as well.

Direct evidence for NK cell involvement in atherogenesis is scant, though some researchers have localized NK cells to the human atherosclerotic plaque. A detailed immunohistochemical analysis of autopsy specimens derived from the PDAY (Pathobiological Determinants of Atherosclerosis in Youth) study did find CD56 staining of NK cells in the intima of early lesions, though these cells were generally low in number and scattered throughout

the lesions, more so in the shoulder regions than the necrotic core (20). Other immunohistochemical studies examining human atherosclerotic lesions contributing to aortic and cerebral berry aneurysms have found NK cells in these plaques and have implicated NK cells in the disease process itself (21, 22). Patients with severe atherosclerotic disease have higher circulating levels of NK cells (23), though a study of elderly patients with peripheral arterial disease found lower NK cell cytotoxicity on a per-cell basis along with a similar trend towards an increased number of total circulating NK cells (24).

As with human atherosclerosis, only a few studies have examined NK cells in the mouse-modeled disease. Immunostaining atherosclerotic lesions from LDL receptor deficient ($LDLR^{-/-}$) mice maintained on a high-fat diet with a carefully titrated asialo-GM1 antibody did show positive staining in early but not late lesions (25). When $LDLR^{-/-}$ mice were crossed with perforin deficient mice, no change in the extent of atherosclerosis was observed, even though NK cell cytotoxicity was impaired. On the other hand, when $LDLR^{-/-}$ mice were crossed with the $Lyst^{beige}$ mutant mice in which the release of proteins from the cytoplasmic granules in NK cells is defective, a significant decrease in atherosclerosis was measured, again in the face of defective NK cell cytotoxicity. When crossing this $LDLR^{-/-}Lyst^{beige}$ model onto a $RAG1^{-/-}$ background, the atherosclerotic burden actually increased, though this was accompanied by an increase in plasma total cholesterol levels as well. Puzzling as this may seem, this finding potentially implicates the $Lyst^{beige}$ mutation as pro-atherogenic in the setting of adaptive immune deficiency, via effects at the vessel wall and/or on lipid metabolism. In other studies using a mouse model of transplant-associated atherosclerosis, it was determined that NK cells were not involved in this process based on observations using $Lyst^{beige}$ mutant mice as recipients (26). In interpreting these studies, it is important to note that NK cells are still present in both the $Lyst^{beige}$ mutant and perforin

deficient models, and despite their defects in granule-mediated target cytolysis, these NK cells may still be capable of producing cytokines that modulate the disease process. This notion fits well with the aforementioned study of NK cells in the elderly, where decreased cytolysis and increased numbers of circulating NK cells correlated with atherosclerosis.

There are a number of chemokines present in the atherosclerotic lesion that may directly influence NK cells. Monocyte chemoattractant protein-1 (MCP-1) functions as a potent chemoattractant for monocytes and T-lymphocytes (27, 28), is found in the atherosclerotic lesion, and has been shown to be chemoattractant for NK cells as well (29). Fractalkine (CX3CL1) is another chemokine found in both human and murine atherosclerotic lesions via immunohistochemistry (30, 31). Its actions include the induction of NK cell migration and activation, leading to increased cytotoxicity and the production of the proatherogenic $\text{IFN}\gamma$ (32). Finally, interleukin-15 (IL-15) is a critical trophic and activating cytokine required for NK cell development. The expression of this cytokine in both human and murine atherosclerotic plaques (33) may contribute to the recruitment, maintenance, and activation of NK cells in the atherosclerotic lesion. In short, these cytokines and others capable of NK cell recruitment and activation are present in the atherosclerotic lesion and their atherogenic potential may partially be linked to NK cell involvement.

Finally, the most direct evidence to date for NK cell involvement in atherosclerosis comes from a recent study using a transgenic model overexpressing the inhibitory Ly49A receptor under the control of the granzyme A promoter (34). In this model, any cell type expressing granzyme A (including NK cells) would also express Ly49A on their cell surface, which would prevent cell activation when the inhibitory Ly49A receptor interacts with MHC class I molecules on the target cell (35). Performing bone marrow transplantation from these

mice into lethally irradiated LDLR^{-/-} recipients resulted in a profound decrease in atherosclerosis without any changes in plasma lipids, implying that NK cells are pro-atherogenic. Though intriguing, the interpretation of these results is complicated, since significant numbers of natural killer T (NKT) cells, CD8⁺ cytolytic T-lymphocytes, and other lymphocytes that have the potential to express granzyme A would also be affected in this system. Regardless, this study supports the notion that NK cells are involved in the pathogenesis of atherosclerosis, though their specific mechanistic role is yet to be determined.

Mast Cells

Another component of the innate immune response that has been implicated in the pathogenesis of atherosclerosis is the mast cell. Mast cells are bone marrow derived cells that reside in connective or mucosal tissues and are involved in inflammation and hypersensitivity reactions. Upon activation, mast cells release the contents of their large cytoplasmic granules that contain a number of biologically active agents: vasoactive substances (histamine and leukotrienes), proteolytic enzymes (tryptase and chymase), inflammatory cytokines (TNF α), and growth factors (PAF). The role of mast cells in atherogenesis is likely to be related to the release of these substances following activation.

Mast cells can be activated in a number of ways. The primary means of degranulation occurs when antigen binds to and crosslinks surface bound IgE. In addition, components of the complement cascade known as anaphylotoxins (C3a and C5a) can activate mast cells. Notably, complement is abundant in the atherosclerotic plaque (36). Aside from these stimuli, direct neural stimulation (37) or excessive cholesterol incorporation into lipid rafts (38) may be

involved in activating mast cells in the plaque as well, though the primary means of mast cell activation in the context of atherosclerosis is still unknown.

Though infrequently found in non-diseased arteries, mast cells are present in human atherosclerotic lesions throughout plaque development, especially in the rupture-prone shoulder regions (39, 40). This anatomical localization highlights their purported role in promoting plaque rupture and subsequent atherothrombotic events. The proteolytic enzymes chymase and tryptase may directly degrade matrix components of the fibrous cap, leading to an unstable plaque phenotype. In addition, these enzymes have been shown to cleave and activate pro-matrix metalloproteinases in carotid artery atherosclerosis, thereby indirectly leading to matrix degradation and plaque instability (41). Finally, heparin proteoglycans and chymase have been shown to inhibit vascular smooth muscle cell proliferation and collagen synthesis *in vitro*, supporting the view that mast cell activation can lead to plaque instability (42).

Mast cell-derived proteases have also been implicated in degrading lipoproteins, leading to aberrant lipoprotein metabolism and atherogenesis. Chymase can degrade HDL associated apolipoproteins involved in reverse cholesterol transport including apoA-I, apoE, and apoA-II (43, 44). More specifically, chymase degradation of these apolipoproteins inhibits ABCA1 mediated cellular cholesterol efflux while leaving SR-B1 mediated and passive diffusion pathways intact (45). Furthermore, chymase can also degrade phospholipid transfer protein, thereby preventing phospholipid transfer to HDL₃ particles and the subsequent formation of pre- β -HDL (46). Mast cell degranulation can also lead to LDL degradation, more specifically apoB-100 proteolysis (47), which can in turn become a nidus for further LDL modifications leading to inflammation and scavenger receptor mediated uptake. Supporting this notion is the finding that

chemical inhibition of chymase was shown to suppress lipid deposition in the aortas of diet-induced hypercholesterolemic hamsters (48).

Mast cell degranulation upon activation releases cytokines and vasoactive substances as well. Immunohistochemical staining of atheromatous coronary arteries show that plaque mast cells contain the pro-inflammatory and atherogenic cytokine $\text{TNF}\alpha$ in pre-formed secretory granules (49). Activated mast cells can regulate T-cell and macrophage responses by secreting a number of other cytokines and inflammatory agents, including MCP-1, macrophage inflammatory proteins ($\text{MIP-1}\alpha$, $\text{MIP-1}\beta$), and various interleukins (IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, and IL-16) (50). Histamine release from activated plaque mast cells constricts muscular arteries which may account for coronary vasospasms that lead to angina or subsequent myocardial infarction (51, 52). Histamine increases endothelial permeability in part by inducing the phosphorylation of vascular endothelial (VE)-cadherin found on vascular endothelial cells (53), which in turn may facilitate the extravasation of lipoproteins and inflammatory cells to atherosclerosis-susceptible regions of the vasculature. On a cellular level, histamine can induce proliferation and matrix metalloproteinase-1 secretion by smooth muscle cells, enhance the expression of adhesion molecules by stimulated endothelial cells, and regulate the Th1/Th2 polarization of T-lymphocytes in the plaque (54). Growth factors such as basic fibroblast growth factor are produced by plaque mast cells and may promote plaque progression and neovascularization (55). Expression of 5-lipoxygenase by plaque mast cells may contribute not only to the production of the inflammatory leukotrienes, but also may lead to oxidation of retained lipids further driving atherogenesis (56). Finally, mast cells appear to be associated with the process of vascular calcification seen in advanced plaques (40). Therefore, there is increasing

evidence that mast cells are not only present in the atherosclerotic plaque, but may also help drive the inflammatory response that characterizes this disease.

Neutrophils

The neutrophil is the most common type of leukocyte found in the circulation and is a major component of the innate immune response. These short-lived phagocytic cells are primarily involved in acute inflammation by engulfing damaged tissue and bacteria, killing invading microbes through the respiratory burst, and secreting proteolytic enzymes such as neutrophil elastase and matrix metalloproteinases. A number of epidemiological and clinical studies have found leukocytosis in general and specifically elevated levels of neutrophils in the circulation to be an independent risk factor for coronary heart disease (57). While neutrophils are generally not detected in stable atherosclerotic plaques, they are prevalent in eroded or ruptured plaques obtained from patients with acute coronary syndromes (58). At this time, it is unclear whether the proteinases secreted by recruited neutrophils lead to plaque erosion and rupture or whether these cells just accumulate at the site of tissue damage, especially since these matrix degrading enzymes are also synthesized by other cell types in the plaque, namely macrophages and smooth muscle cells (59-61) and possibly mast cells as well. Activated neutrophils also secrete myeloperoxidases which may contribute to atherosclerosis by oxidizing LDL leading to uptake by macrophages (62) as well as by modifying apoA-I thereby attenuating ABCA1-dependent cholesterol efflux (63).

Cells that Bridge the Innate and Adaptive Immune Responses

Dendritic Cells

As previously discussed, the inflammatory response to retained and modified lipids in the vessel wall is the hallmark of the atherosclerotic lesion. An important initial step in this inflammatory cascade is the processing and proper presentation of the putative plaque antigens to the T-lymphocytes that participate in atherogenesis. Recent work has shown that the dendritic cell may be an important regulator of this inflammatory response by acting as an efficient antigen presenting cell.

Dendritic cells initially described by Steinman and Cohn (64) are professional antigen presenting cells that are able to initiate primary immune responses. Although dendritic cells as a family are heterogeneous and functionally diverse, these bone marrow derived cells arise from a common CD34⁺ progenitor and progress functionally through different stages of development. Dendritic cells are able to regulate the immune response to foreign and self-antigens, and therefore are important in either initiating an adaptive immune response or conversely by inducing tolerance (65). To generalize dendritic cell maturation, immature dendritic cells efficiently sample their antigenic microenvironment through macropinocytosis and receptor mediated endocytosis. Activating signals transmitted largely by the diverse complement of Toll-like receptors expressed by dendritic cells induce maturation (66). This functional change is characterized by a downregulation of the endocytic machinery and an upregulation of the expression of antigen-presentation molecules (MHC I and II, CD1), co-stimulatory molecules (CD40, CD80/B7.1, CD86/B7.2), and the secretion of inflammatory cytokines (such as IL-12 and TNF α) (66). This phenotypic switch facilitates interaction with and subsequent activation of T-lymphocytes, thereby initiating the adaptive immune response (67).

Dendritic cells are a component of the proposed vascular associated lymphoid tissue (VALT) and are found in the intima of susceptible arteries prior to atherosclerotic lesion

development (68). Monocyte precursors and dendritic cells are also recruited to the growing plaque by the activated endothelium throughout atherogenesis (69). Dendritic cells have been identified in the atherosclerotic plaques of both humans and mice by immunohistochemical and PCR based approaches, implicating them in the pathogenesis of the disease (70-72). Dendritic cells colocalize with T-lymphocytes in the shoulder regions of rupture prone atherosclerotic plaques (73), suggesting that antigen presentation and co-stimulation by plaque dendritic cells leads to activation of T-lymphocytes at this site which may ultimately contribute to plaque destabilization and subsequent atherothrombosis. Dendritic cells in the vessel wall also express components of the complement system, namely C1q, which likely facilitates the capture of immune complexes in the atheroma (74).

Modified lipids present in atherosclerosis have been shown to influence dendritic cell maturation and activation in vitro. Oxidized low density lipoprotein (oxLDL) not only promotes monocyte to dendritic cell maturation (75), but also increases the expression of antigen presenting and co-stimulatory molecules on the mature dendritic cell (76). One of the modified lipids generated during the oxidative process is lysophosphatidylcholine, which may induce dendritic cell maturation directly through G-protein coupled receptor signaling (77) or indirectly by preventing the maturation block mediated by PPAR γ signaling (78). The liberation of these signaling phospholipids by secretory phospholipase A₂ has also been recently implicated in dendritic cell maturation (79). On the other hand, the maturation and antigen-presentation ability of dendritic cells can be inhibited by statins (80), the cholesterol-lowering class of drugs that has been found to have a number of immunomodulatory effects as well. Dendritic cells treated with polyunsaturated fatty acids of the n-3 and n-6 family also display a maturation block when challenged with lipopolysaccharide, suggesting that part of the reported anti-inflammatory and

atheroprotective effect of these dietary fatty acids (81, 82) may be linked to preventing dendritic cell activation (83). Finally, recent studies have identified other lipid mediators that may be responsible for retaining dendritic cells in the atherosclerotic plaque, namely platelet activating factor (PAF) and 18:1 lysophosphatidic acid (84, 85). By preventing dendritic cells from leaving the plaque, these lipid mediators contribute to both continued plaque growth as well as the lesional localization of the inflammatory response to plaque antigens. Interestingly, HDL-associated platelet activating factor acetylhydrolase (PAF-AH) can inhibit this dendritic cell retention (85), highlighting another atheroprotective attribute of HDL.

Other recognized risk factors for atherosclerosis may also directly influence dendritic cell biology in the plaque. Nicotine as a major component of cigarette smoke is able to dose-dependently activate dendritic cells leading to increased Th1 cytokine secretion by T-cells (86). On the other hand, the non-enzymatic glycation of proteins that occur in the setting of diabetes appears to promote dendritic cell maturation, but prevents their expression of co-stimulatory molecules and their ability to activate T-cells (87). Finally, dendritic cells may play an important role in the inflammation induced by infectious agents in the vessel wall, another potential contributor to atherogenesis (65, 88).

$\gamma\delta$ T-Lymphocytes

T-cells are members of the adaptive immune response that respond to specific antigens that complement their rearranged T-cell receptor (TCR). Although the majority of T-lymphocytes express the $\alpha\beta$ TCR, another subset of T-cells exist that bear the $\gamma\delta$ type of TCR. These cells represent less than 5% of the T-cell population in the peripheral human blood, though are enriched in specific tissues, including the gastrointestinal mucosa, skin, and splenic pulp as

well as at sites of chronic inflammation like the joint synovium in rheumatoid arthritis (89). $\gamma\delta$ T-cells have a limited TCR diversity, are thought to be important for the initial defense against epidermal and mucosal pathogens, and can either activate or suppress other lymphocyte subsets (90). This immunomodulatory function stems largely from the cytokines they produce, which include both Th1 and Th2 types; namely IL-2, IL-4, IL-5, IL-10 and IFN γ (91). In contrast with traditional $\alpha\beta$ T-cells, some $\gamma\delta$ T-cells do not require antigen processing and presentation in the context of MHC molecules in order to respond to their cognate antigen (91). This unique property of $\gamma\delta$ T-cells to rapidly respond to free antigen positions these cells as bridging lymphocytes between the innate and adaptive immune responses. $\gamma\delta$ T-cells have been detected in the intima of human atherosclerotic lesions, especially in the early stages of lesion formation (20, 92). In the lesions of LDLR^{-/-} and apoE^{-/-} mice, transcripts for the γ -TCR chain have been identified using laser capture microdissection with subsequent RNA isolation and RT-PCR analysis (VanderLaan et al. unpublished data). The role of $\gamma\delta$ T-cells in atherosclerosis remains unclear, but overall these cells may be pro-atherogenic, since the absence of $\gamma\delta$ T-cells led to a 15% reduction in plasma total cholesterol levels and a 21% reduction in aortic sinus atherosclerosis in 18 week old apoE^{-/-}/TCR δ ^{-/-} mice, though these differences did not achieve statistical significance (93).

Natural Killer T-Lymphocytes

It is becoming very clear that the chronic inflammation of atherosclerosis encompasses components of both innate and adaptive immunity (15). To understand this interconnection, one cell type in particular warrants further investigation, namely the natural killer T (NKT) lymphocyte. NKT cells are a subset of lymphocytes characterized by the co-expression of both

NK cell markers (NK1.1/CD161) and a functional TCR complex. The most intriguing property of NKT cells from the standpoint of atherosclerosis researchers is their ability to recognize lipid and glycolipid antigens presented on CD1d molecules by their semi-invariant TCR (predominantly V α 14J α 18/V β 8 for mice and V α 24J α 18/V β 11 for humans). CD1 molecules are a family of non-polymorphic cell surface glycoproteins expressed by certain antigen presenting cells and have structural and functional similarities to MHC proteins (94). Although a number of bacterial glycolipids such as phosphoinositol mannosides, lipoarabinomannan, mycolic acids, and hexosyl-1-phosphoisoprenoids have been found to be presented by CD1 molecules, endogenous antigens for CD1d remain largely unknown (95), though recently Bendelac and colleagues have identified the lysosomal glycosphingolipid isoglobotrihexosylceramide (iGb3) as a potential endogenous ligand for both human and murine NKT cells (96). Experimentally, this lack of activating endogenous antigens has been bypassed by using the synthetic ligand α -galactosylceramide (α -GalCer) derived from marine sponges to study NKT cell physiology. α -GalCer specifically and robustly activates NKT cells in a CD1d dependent manner when processed and presented by antigen presenting cells (97).

Because NKT cells share many characteristics of both NK cells (innate immunity) and T-cells (adaptive immunity), this lymphocyte in particular is positioned as an immunomodulator: bridging the gap between these distinct phases of the early and late immune response. This cross-talk between NKT cells and other lymphocytes has been previously described. In mice, specific activation of NKT cells resulted in concomitant cytokine production by NK cells and the expression of the activation marker CD69 by NK cells, B-lymphocytes, and CD8⁺ T-lymphocytes *in vivo* (98). Furthermore, NKT cells have also been shown to directly promote B-lymphocyte proliferation and antibody production *in vitro* (99, 100). In all cases, these effects

were found to be CD1d dependent, highlighting the importance of the CD1d-TCR interaction in NKT cell activation.

Since one of the earliest events in the pathogenesis of atherosclerosis is the retention and subsequent oxidative modification of lipids and lipoproteins in the vessel wall, it follows that the NKT cell may be involved in reacting to these lipid neoantigens in the plaque when presented on CD1 molecules by either macrophages or dendritic cells. In fact, glycosphingolipids and gangliosides have been identified in human atherosclerotic tissue, and increases in plasma cholesterol levels are associated with elevated glycosphingolipids as well (101-103). In apoE^{-/-} mice, multiple gangliosides have been extracted from the diseased vessel wall, and plasma levels of gangliosides were increased 7-fold as compared to wild-type controls (101). Therefore, it is attractive to hypothesize that NKT cells may be activated by modified lipid antigens either present in or induced by oxidized lipoproteins.

Direct evidence for NKT cell involvement in atherosclerosis is limited to a handful of studies. In human atherosclerotic lesions, macrophage foam cells have been shown to strongly express all four human CD1 proteins (CD1a, b, c, d) (104). In patients with both stable and unstable angina, a decrease in the number of circulating NKT cells was observed. One possible explanation for this finding is that once the NKT cells are activated, they secrete IFN γ and subsequently undergo apoptosis, thus accounting for the decreased numbers of these cells in the circulation (105). On the other hand, it has been recently appreciated that activation of NKT cells does not necessarily lead to apoptosis, but can instead result in a downregulation of the TCR and NK1.1/CD161 to prevent over stimulation (106). In the lesions of LDLR^{-/-} and apoE^{-/-} mice, transcripts for the semi-invariant TCR (V α 14J α 18) have been identified using laser capture microdissection with subsequent RNA isolation and RT-PCR analysis (VanderLaan et al.

unpublished data). In apoE^{-/-} mice, exogenous administration of LPS increased both the extent of atherosclerotic lesions and the numbers of circulating and plaque NKT cells (107). Exogenous administration of α -GalCer increased atherosclerosis in apoE^{-/-} mice, whereas CD1d deficient mice showed reduced atherosclerotic lesion development (108-110). This α -GalCer driven increase in atherosclerosis was accompanied by a dramatic release of both the atherogenic cytokine IFN γ as well IL-4 by NKT cells (110). Interestingly, it has recently been shown that a gender dimorphism exists with respect to α -GalCer induced cytokine secretion, with significantly higher levels of IFN γ achieved in the serum of females versus males, whereas there was no difference in IL-4 levels (111). *In vitro* experiments have shown that macrophages incubated with oxidized LDL display increased expression of CD1d, which can in turn induce NKT cells to produce IFN γ (109). Finally, a recent study not only suggests that NKT cell involvement in atherogenesis is mostly limited to the early fatty streak lesions with little effect on larger and more advanced lesions, but also found that the absence of NKT cells did not significantly alter cytokine mRNA levels in the vessel wall (112). An important point to note here is that all these aforementioned experiments were performed in the presence of an otherwise functional immune system, and therefore the pro-atherogenic potential of NKT cells may be due to their interactions with other lymphocytes present either in the plaque itself or else in other lymphoid compartments. In any case, at this time it appears that CD1d-mediated activation of NKT cells results either directly or indirectly in creating an inflammatory cytokine expression profile that drives the progression of atherosclerotic lesion development.

Cells of the Adaptive Immune System

Regulatory T-Lymphocytes

Regulatory T-lymphocytes (T_{reg}) are a somewhat heterogeneous subset of $CD4^+$ T-cells that can suppress inflammation and induce tolerance, thereby modulating the adaptive immune response (113). Through either cytokine secretion (including IL-10 and $TGF\beta$) or through direct cellular interactions, T_{reg} cells can exert their immunosuppressive functions by inhibiting the proliferation of naïve T-cells as well as reducing both Th1 and Th2-biased responses. In a recent study, ovalbumin-specific T regulatory type 1 cells (Tr1) were generated *in vitro* and adoptively transferred into apoE^{-/-} mice that were also immunized with ovalbumin (114). This resulted in a reduction in the amount of atherosclerosis in the thoracic aorta and the aortic sinus without any change in plasma cholesterol levels. In addition, the composition of the aortic sinus lesions was altered, with a reduction in the number of macrophages and T-cells but not smooth muscle cells. Intense IL-10 staining was detected in the aortic sinus lesions via immunohistochemistry. However, it is not clear if these lesion differences were mediated directly by IL-10 or rather indirectly due to the suppression of both Th1 and Th2 responses, as suggested by the decreased production of both the Th1 cytokine $IFN\gamma$ and the Th2 cytokines IL-4 and IL-5 by T-cells isolated from the adoptively transferred mice. Whatever the case may be, the ability of these cells to modulate the immune response makes them attractive therapeutic targets in preventing atherosclerosis.

B-Lymphocytes

Experimentally, B-cells as a group have been shown to be atheroprotective, since eliminating them either genetically (9) or through splenectomy (10) increases atherosclerosis. Although B-lymphocytes are generally not detected in atherosclerotic lesions (115, 116), an adventitial localization of these cells may partially explain their demonstrated influence on

plaque development (117, 118). On the other hand, the major immunological product of B-cells, namely immunoglobulins, is readily identified in the plaque throughout lesion development (119, 120). Antibodies that recognize oxLDL have been found in the circulation of both humans and mice (121, 122). This has recently focused attention on the role that a sub-population of innate B-cells may play in atherogenesis, namely B-1 B-lymphocytes, which will be extensively covered by Witztum and colleagues in a separate review of this series. In total, most studies to date suggest that B-lymphocytes in general are anti-atherogenic on the basis of the protective antibodies they produce, but it is important to consider other roles that B-cells may play in modulating the atherosclerotic immune response, namely through antigen presentation and cytokine secretion.

B-cells are able to selectively internalize their cognate antigen via the B-cell receptor (BCR), which make B-cells extremely efficient at presenting their respective antigen to T-cells. B-cells also express co-stimulatory molecules, enabling them to activate T-cells that also recognize the same antigen, thereby initiating and amplifying the immune response (123). For this reason, B-cells should be considered along with the other antigen presenting cells involved in atherosclerosis, namely macrophages and dendritic cells. B-cells isolated from the spleens of aged apoE^{-/-} mice with substantial atherosclerotic lesions had a significantly increased expression of the co-stimulatory molecules CD80/B7.1 and CD86/B7.2 as compared to B-cells from either younger apoE^{-/-} mice devoid of atherosclerosis or age matched C57BL/6 controls (124). Furthermore, increased numbers of circulating activated B-cells expressing CD80 positively correlate with the severity of carotid atherosclerosis as assessed by intima-media thickness (IMT) using high-resolution ultrasonography (125). Finally, certain subsets of B-cells are characterized by increased CD1d expression (126), which implies that certain B-cells could potentially present

lipid antigens to NKT cells, though this intriguing link to atherogenesis has not yet been demonstrated.

In addition to their ability to produce antibodies and present antigen to T-cells, it has been recently appreciated that B-cells can regulate the immune response directly through cytokine secretion as well. Under certain conditions, B-cells are able to produce a variety of cytokines once thought to be restricted to T-cells, including IL-1, IL-2, IL-4, IL-6, IL-10, IL-12, IL-13, IL-16, IFN γ , lymphotoxin (LT) α and β , TGF β , and TNF α (124). The particular cytokines produced by any given B-cell appears to be context dependent, influenced by both the local cytokine milieu and any stimulatory signals (such as through the BCR or CD40) (127). Distinct subsets of cytokine secreting B-cells have been recently identified. Effector B-cells (Be1 and Be2) secrete either type 1 cytokines (IFN γ , IL-12, and LT α) or type 2 cytokines (IL-2, IL-4, IL-6) respectively (123, 128), analogous to the Th1 vs. Th2 polarization paradigm of T-lymphocytes. Regulatory B-cells (B_{reg}) are similar to T_{reg} in that they primarily produce the anti-atherogenic cytokine IL-10, which can suppress inflammation and inhibit the Th1-biased response (123). Interestingly, B-1 cells are a major source of B-cell derived IL-10 (129), which may implicate this subset of B-cells in helping to suppress the inflammatory response to oxLDL in atherogenesis. While intriguing and potentially important in the pathogenesis of atherosclerosis, there is currently a paucity of studies that examine the specific role of cytokine production by B-cells in this disease process.

Conclusions

Atherosclerosis is indeed a complex inflammatory disease, and this review has focused on a number of cell types that participate in this inflammatory response. As mentioned before,

the macrophage foam cell, fibro-proliferative vascular smooth muscle cell, and the vascular endothelial cell constitute the major cell types involved in this disease, and really should still be regarded as the “usual suspects” in atherogenesis. Despite the emerging roles that other inflammatory cells and regulatory lymphocytes may play in atherogenesis, it is important to keep in mind that these cells are quantitatively minor components of lesions and by themselves are probably not sufficient for the development of atherosclerosis, but rather appear to modulate the course of the disease. That said, the roles played by these “*unusual suspects*” are becoming increasingly important in understanding the complexity of this disease process that encompasses hemodynamics and biorheology, lipid and lipoprotein metabolism, coagulation and hemostasis, and finally innate and adaptive immunity. The immune networks at play are recognized to be increasingly complex, with multiple cellular and molecular interactions dictating the characteristics of the inflammatory response. Thus far, knockout and transgenic studies in mice have proved extremely useful in delineating the atherogenicity of each of these particular cell types; affecting atherosclerosis through both general immune responses as well as more plaque specific immune responses. Looking forward, these “*unusual suspects*” may become even more prevalent when dissecting the complex cellular interactions that occur in the context of atherosclerosis, and may prove to be useful therapeutic targets in preventing the clinical complications of this disease process.

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