ASIP CENTENNIAL REVIEW

Adaptive Immunity and Atherosclerosis

Mouse Tales in the AJP

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Chronic inflammation driven by immune responses to lipid deposition in the arterial wall is now understood to be fundamental to the pathogenesis of atherosclerosis. The frequent presence of T lymphocytes in human atherosclerotic lesions was first described in the 1980s, but experiments to test whether adaptive immunity influences lesion development and phenotype required animal models. *The American Journal of Pathology* has published many research articles focused on the role of inflammation and adaptive immunity in diet-induced and genetically manipulated murine models of atherosclerosis. Seminal articles in the 1990s were the first to describe the presence of T cells in mouse atherosclerotic lesions; other articles demonstrated the effects of defective adaptive immunity on lesion development in mice. *(Am J Pathol 2013, 182: 5—9; http://dx.doi.org/10.1016/j.ajpath.2012.10.006)*

The pathogenesis of atherosclerosis involves chronic inflammatory responses to lipoproteins deposited in the arterial intima. The essential importance of inflammation to this disease is clearly evident from analyses of human and animal lesions, and from experimental manipulation of animal models. A previous review in *The American Journal of Pathology* (*AJP*) discusses the possible causes and consequences of inflammation in the context of atherosclerosis.[1] In the 1980s, Goran Hansson and colleagues[2,3] first described the regular presence of activated T lymphocytes in human atherosclerotic plaques, and similar findings were reported by Emeson and Robertson.[4] These studies were among the first to indicate that adaptive immune responses could be involved in the pathogenesis of atherosclerotic lesions. At that time, inbred strains of mice had already been established as essential tools for immunology research, and so the potential usefulness of mice to experimentally examine the influences of the immune system on atherosclerosis was an obvious consideration after the provocative, albeit descriptive studies implicating T cells in human arterial disease. Seminal studies that focused on immune modulation of atherosclerosis in mice, some of which were published in the *AJP*, represent the dawn of a highly productive era of mouse-based research in cardiovascular immunology. This review discusses progress in our understanding of the influences of adaptive immunity

in atherosclerosis, with a focus on T cells, as reflected by *AJP* articles over three decades.

Seminal Studies with C57BL/6 Mice in the *AJP*

Before robust genetically manipulated mouse models of atherosclerosis became widely available, investigators studied this disease in C57BL/6 mice a fed high-fat, cholesterol-rich diet containing cholic acid (known as the Paigen diet), which reliably induced lipid-rich, fatty streak lesions in the aortic root.[5] In the first study attempting to determine how T cells influence atherosclerosis, Emeson and Shen[6] examined the effects of cyclosporin treatment on lesion development in C57BL/6 mice fed a Paigen diet and reported their findings in the *AJP*. Contrary to expectations, the mice receiving the calcineurin inhibitor developed bigger lesions. That study failed to determine whether T cells had direct effects on lesion development because the drug treatment resulted in markedly elevated blood levels of total and low-density lipoprotein cholesterol, reflecting the effects of calcineurin inhibitors on several cell types. Although in that study the authors did not clarify how adaptive immunity contributes to atherosclerotic disease, they did establish the

general approach of monitoring lesion development in mice after pharmacological immune intervention. Importantly, the article highlighted the need to rule out effects of the interventions (pharmacological or genetic) on lipid metabolism before making conclusions about direct effects of immune responses on lesion development.

Three years later, Emeson et al[7] published a second study in the *AJP*, in which they examined the influence of T-cell deficiency on aortic root lesion development in cholesterol/cholic-acid—fed mice. In that study, the investigators fed an atherogenic diet to T-cell—deficient *nu/nu* or control *nu/+* mice for approximately 31 weeks. They reported a marked reduction in aortic root lesion size in the T-cell—deficient group. At that time in the 1990s, there were general concerns in the field about the relevance of lesion development in the cholic-acid—fed C57BL/6 mouse model, because of systemic and hepatic inflammatory effects of the diet and because of the lack of development of mature lesions with smooth muscle cells and fibrosis. In retrospect, however, the results actually portended the similar findings that would be seen with global adaptive immune deficiency in mouse atherosclerosis models that do not require cholic acid. Emeson et al[7] also found that depletion of CD4^{+} cells using monoclonal antibodies reduced aortic lesion size by approximately 70%. This result was also predictive of later studies demonstrating proatherogenic effects of CD4^{+} T cells, using mice genetically susceptible to atherosclerosis.[8,9]

Adaptive Immunity and Genetically Hypercholesterolemic *Apoe*^{−/−} and *Ldlr*^{−/−} Mice

In the early 1990s, the *Apoe*^{−/−} and *Ldlr*^{−/−} mouse strains were derived by homologous recombination-mediated gene deletion; these animals develop severe hypercholesterolemia and atherosclerotic lesions with features of mature human lesions.[10−13] Over the past 20 years, these mouse strains have been widely distributed and extensively used to study many aspects of dyslipidemia and atherosclerosis. In one of the first reports of using such mice to study adaptive immunity in atherosclerosis, Hansson and colleagues[8] identified CD4^{+} T lymphocytes in early and advanced lesions of *Apoe*^{−/−} mice fed either a standard or cholesterol/high fat diet. The T cells were found to express the activation marker CD25.[8] The authors also found abundant class II MHC expression in the lesions, suggesting local immune activation and indicating that antigen presentation to CD4^{+} T cells within plaques is possible. This article, published in the same issue of the *AJP* as the above-mentioned study of Emeson et al,[7] was important because it showed similarities in the immune phenotype of lesions in the *Apoe*^{−/−} model and human lesions. In the same month, the presence of T cells in lesions of both *Ldlr*^{−/−} and *Apoe*^{−/−} mice was also reported by Daugherty and colleagues.[14] The knowledge that atherosclerotic lesions in these mouse strains, like human lesions, contain the cellular and molecular signature of the effector phases of T-cell—mediated immune responses was key for stimulating future investigative work on the mechanisms and effects of these responses.[15]

In another early study published in the *AJP* addressing the influence of adaptive immune responses to atherosclerosis, Robert Colvin and colleagues[16] transplanted hearts from donor mice of the 129 strain into *Apoe*^{−/−} C57BL/6 or control C57BL/6 mice. They observed that in the hyperlipidemic *Apoe*^{−/−} recipients, lipid-rich aortic and coronary atherosclerotic lesions in the transplanted hearts were more severe than in the native hearts, with more infiltrating lymphocytes, indicating that T-cell—mediated alloreactivity enhanced atherosclerosis.

The Effects of Immunodeficiency on Atherosclerosis in *Apoe*^{−/−} and *Ldlr*^{−/−} Mice

The development of the robust *Apoe*^{−/−} and *Ldlr*^{−/−} mouse models permitted investigators to test the effects of different components of the immune system by crossbreeding these mice with other strains carrying null mutations in immunologically relevant genes. Some of the first studies taking this approach addressed the question of the effect of global loss of the entire adaptive immune system. *Rag1*^{−/−} or *Rag2*^{−/−} mice lack the V(D)J recombinase required to form lymphocyte antigen receptor genes. Therefore, *Rag1*^{−/−} or *Rag2*^{−/−} mice have a complete absence of B and T cells; that is, they have no adaptive immune system. SCID mice carry mutations in a gene encoding a DNA repair enzyme that is needed for antigen receptor gene formation, and these mice have a severe, albeit not complete, lack of T and B cells. When *Apoe*^{−/−} mice were fed a high-fat, high-cholesterol diet, resulting in high plasma cholesterol concentrations (~ 1000 mg/dL), there was little effect of RAG deficiency (ie, lymphocyte deficiency).[17,18] In *Apoe*^{−/−} mice fed a regular chow diet, however, with plasma cholesterol concentrations of approximately 390 to 470 mg/dL, lymphocyte deficiency resulted in a 40% reduction in lesion formation.[17] *Apoe*^{−/−} SCID mice fed a regular chow diet showed a 73% reduction in aortic lesions, compared with immunocompetent *Apoe*^{−/−} mice fed the same diet; plasma cholesterol content in both groups was approximately 470 mg/dL.[19] RAG-deficiency in atherogenic diet fed *Ldlr*^{−/−} mice resulted in significantly reduced early lesion development compared to immunocompetent *Ldlr*^{−/−} mice.[20] The serum cholesterol levels in the *Ldlr*^{−/−} mice in that study were approximately half the levels usually attained in similarly fed *Apoe*^{−/−} mice.

Overall, studies with mice lacking an adaptive immune system suggest a significant proatherogenic role for the adaptive immune system; moreover, the effect can be obscured when hypercholesterolemia is severe. Given that one central ultimate effector of adaptive immunity is the activated macrophage, and macrophages can be activated in the absence of T cells or antibodies by innate stimuli generated by oxidative degradation of low-density lipoprotein, it

is not surprising that the influence of the adaptive immune system on atherosclerosis is not apparent under extreme hypercholesterolemic conditions.

Comparison of atherosclerotic lesion development between atherosclerotic-prone ($Apoe^{-/-}$ or $Ldlr^{-/-}$) mice with or without homozygous null mutations of Rag or $Scid$ genes may seem like a straightforward way to determine whether adaptive immunity does or does not influence atherosclerosis. However, atherosclerosis and the immune system are too complex for such a simple approach to always yield readily interpretable information. In fact, the net effect of the global loss of all components of the adaptive immune system on atherosclerotic disease in these mouse models will reflect both a loss of proinflammatory effector functions (T cells, B cells, and antibodies) and a loss of regulatory functions of T and B cells that affect endothelium and innate immune system cells. There is also solid evidence for direct atheroprotective effects of some, but not all, antibodies induced under hypercholesterolemic conditions.[21] Thus, more selective manipulations of distinct components of the adaptive immune system are necessary for an understanding of their roles in promoting or protecting against atherosclerosis.

Indeed, when more selective approaches have been taken, profound effects of T-cell and B-cell−mediated immunity on atherosclerosis become evident; these effects have been summarized in many reviews, including that of Hansson et al.[22] This literature includes two reports, published in the *AJP*, of studies by Elhage et al,[9,24] who used several compound mutant mice that are susceptible to hypercholesterolemia and also are selectively immunodeficient. In one study, these investigators compared lesion development in $Apoe^{-/-}$ mice with null mutations of αβ T-cell receptors (TCRαβ; the type expressed by most T cells), TCRγδ, CD4, or CD8.[9] Their work showed a profound reduction in disease in the absence of all TCRαβ T cells; interestingly, it also showed a significant regional increase in disease in the absence of just CD4+ T cells.[9] In retrospect, the latter result may reflect the loss of CD4+ regulatory T cells (Tregs), in addition to effector CD4+ T cells, leading to enhanced responses by CD8+ T cells and innate effectors. The importance of Tregs in atherosclerosis has become apparent from more recent studies in mouse models (reviewed by Nilsson et al[23]).

In the other study, Elhage et al[24] determined that the protective effects of estradiol against fatty streak formation in $Apoe^{-/-}$ and $Ldlr^{-/-}$ mice are lost in RAG-2−deficient $Apoe^{-/-}$ and $Ldlr^{-/-}$ mice and are restored by reconstitution with $Rag2^{+/+}$ bone marrow. Furthermore, they showed that $Apoe^{-/-}$ mice with selective deficiencies in just conventional TCRαβ T cells, CD4+ T cells, CD8+ T cells, or B cells all exhibited lesion reduction by estradiol similar to that seen in immunocompetent $Apoe^{-/-}$ mice.[24] These studies demonstrate the power of analysis of compound mutant mice in evaluating the influence of different components of the adaptive immune system on atherosclerosis, and they

highlight the presence of both lymphocyte subset-specific effects and shared effects.

Helper T-Cell Subsets and Cytokines

Since the 1980s, the known landscape of cells and molecules that mediate and regulate adaptive immune responses has steadily grown more complex. One of the emerging complexities is the presence of multiple subsets of CD4+ T cells. T helper type 1 cells (Th1), which produce interferon γ (IFN-γ), and Th2 cells, which produce IL-4, IL-5, and IL-13, were first described in the 1980s,[25] and more recent work has established the existence of Th17 cells, which produce IL-17 and IL-22.[26] Each of these helper T-cell subsets plays distinct roles in microbial defense and in disease. Furthermore, as noted above, Tregs are a subset of CD4+ T cells that play critical roles in preventing autoimmunity and excessive immune responses to exogenous antigens.[27] $Apoe^{-/-}$ and $Ldlr^{-/-}$ mice have served as key tools for investigating whether and how these various CD4+ T-cell−subsets affect atherosclerosis. For example, studies with $Ldlr^{-/-}$ mice also carrying null mutations of the genes encoding IL-18,[28] IFN-γ receptor,[29] IFN-γ,[30] or the Th1 lineage-defining transcription factor T-bet[31] have shown that Th1 responses are the major proatherogenic helper T-cell subset in mice.[29,30] These findings correlate with the presence of IFN-γ and activated CD4+ T cells in human lesions.[32,33] In contrast to Th1 cells, Tregs suppress atherosclerotic lesion growth and inflammation in $Apoe^{-/-}$ and $Ldlr^{-/-}$ mice.[23] Several articles published in the *AJP* have addressed the role of T helper subsets or the cytokines they express or depend on in the $Apoe^{-/-}$ and $Ldlr^{-/-}$ mouse models, including IFN-γ,[34] IL-4, and IL-12.[35] In another *AJP* article, Schulte et al[36] examined the effect of genetic bias of helper cell subset polarization on atherogenesis. Comparing C57BL/6 $Apoe^{-/-}$ and BALB/c $Apoe^{-/-}$ mouse strains, they showed significantly more lesion development in the Th1-biased C57BL/6 background.

Cytokines may also have profound anti-inflammatory and atheroprotective properties, which are indicated by accelerated disease when the cytokines are absent. This was first experimentally demonstrated by genetic ablation of IL-10 in the C57BL/6 model in a study by Mallat et al,[37] and by reconstitution of $Ldlr^{-/-}$ mice with $Il10^{-/-}$ bone marrow in another study from that research group.[38] Hansson and colleagues[39] studied the effect of another important anti-inflammatory cytokine, TGF-β, specifically on T-cell−mediated inflammation in atherosclerosis. They showed that $Apoe^{-/-}$ mice with T-cell−restricted expression of a dominant negative TGF-β receptor had markedly enhanced lesion development and inflammation, compared with $Apoe^{-/-}$ controls. In a subsequent study published in the *AJP*, Hansson and colleagues[40] showed that deposition of mature collagen in lesions was impaired in the $Apoe^{-/-}$ mice with TGF-β−resistant T cells, suggesting that T-cell−mediated inflammation can reduce

plaque stability and enhance vulnerability to rupture. These studies on the effects of IL-10 and TGF-β were important stimuli for investigators to address the role of Tregs in atherosclerosis, because both cytokines are produced by Tregs.

T-Cell Costimulatory Molecules Affect Atherosclerosis

Mouse models of atherosclerosis have also been key in defining roles for T-cell costimulatory molecules in proatherogenic immune responses. Costimulators are molecules expressed on antigen-presenting cells in response to infection or tissue damage and they are required, in addition to antigen recognition, for the activation of naïve T cells to initiate T-cell immune reactions. The best defined and arguably the most important costimulatory molecules are CD80 (B7-1) and CD86 (B7-2), both of which are expressed on dendritic cells and bind to CD28 on T cells. One of the first demonstrations of the importance of costimulation in vascular pathology came from a study of graft arterial disease in class II MHC mismatched cardiac transplants in mice, described in an *AJP* article by Mitchell and colleagues.[41] In that study, the investigators discovered that graft arterial disease was significantly attenuated in $Cd80^{-/-}$ recipient mice, compared with wild-type recipient mice. Although graft arterial disease is not dependent on cholesterol/lipoprotein deposition, it shares several common elements of vascular pathology with atherosclerosis, including accumulation of intimal smooth muscle cells; graft arterial disease can also be accelerated and complicated by hypercholesterolemia.[16] The work from Mitchell's research group[41] served as an important stimulus for studies of the effect of costimulatory molecule deficiency in atherosclerosis using the $Ldlr^{-/-}$ model. These studies have shown that B7 and TNF family costimulators and their CD28 and TNFR family receptors on T cells have profound effects on atherosclerosis in $Ldlr^{-/-}$ and $Apoe^{-/-}$ mice,[32] as does the coinhibitory PD-L1/PD-1 pathway.[42] Drugs targeting CD80 and CD86, CLTA-4, and PD-1 are approved or in advanced clinical trials for treatment of autoimmune diseases or cancer; therefore, it may be soon possible to assess the effects of these target molecules in human atherosclerosis.

Conclusions

Mouse models remain centrally important to research at the intersection of cardiovascular disease and immunology. The approaches represented by the *AJP* articles reviewed here have evolved as technology has advanced, and studies today often include inducible and cell lineage-specific manipulation of genes in a particular immune or vascular cell type. Nonetheless, all animal models have the general limitation that they imperfectly reflect human pathobiology, and this is certainly true for mouse atherosclerosis and mouse immunopathology. One important example is the fact that the mouse models do not adequately recapitulate the human

vulnerable plaque and plaque rupture, but there is abundant circumstantial evidence that inflammation and adaptive immune responses contribute to these processes. The greatest challenge is to identify the findings from mouse studies that are most relevant to human disease, and to move to truly translational studies that can lead to more effective therapies. Given the record of excellence of the *AJP* in reporting advances in the study of human disease, we can look forward to reading in this journal about new important results of human cardiovascular immunology research.

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