

11 Lymphocytes in atherogenesis

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11.1 Introduction

Atherosclerosis is increasingly described as an inflammatory disease.⁽¹⁾ Many descriptions of the inflammatory nature of the disease process have focused on the role of innate immunity through infiltration of macrophages. However, while it has been known for many years that lymphocytes are present within lesions, only recently has there been a wider appreciation for a role of adaptive immunity in the atherogenic process. This recognition is largely based on the presence of activated lymphocytes within lesions that penetrate the vasculature at all stages of the atherogenic process.

In this brief and selective review, we will initially provide a background on the nature of lymphocyte infiltration in atherosclerosis and define features that have been mimicked in animal studies. Subsequently, we will review studies in which lymphocyte function has been regulated by a number of techniques in order to define an effect of adaptive immunity on development of atherosclerotic lesions. Finally, there is a highly selective list to illustrate the complex array of effects that can be exerted on the disease process by cytokines secreted during lymphocyte activation.

11.2 Overview of lymphocytes

Lymphocytes display considerable diversity. The major classes of B and T lymphocytes, their differentiation markers, and examples of the spectrum of cytokines

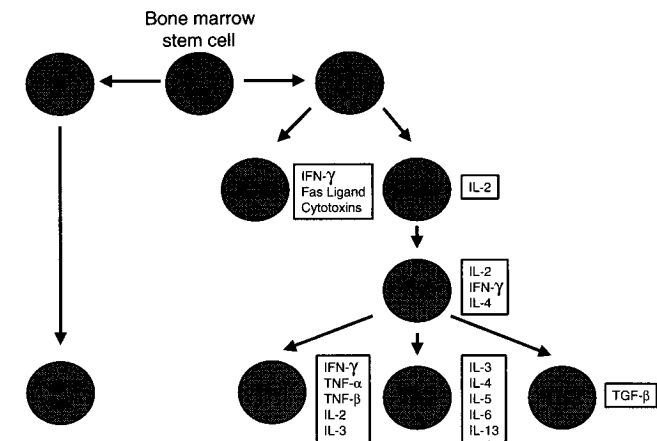


Fig. 11.1 Major classification of lymphocytes, their major role, markers, and selected cytokines secreted by specific populations.

released on activation are summarized in Fig. 11.1. The major subclassification of lymphocytes are B and T cells that function in adaptive immunity, and natural killer (NK) cells that participate in innate immune responses. B and T cells account for humoral and cellular adaptive immune responses, respectively, but there is significant cross-talk between the two systems of cells.

The B cell is activated when a specific antigen ligates its antigen receptor, which is an immunoglobulin inserted into the plasma membrane. Binding of antigen induces a process of differentiation into a B lymphoblast (or activated B cell), which produces large amounts of IgM antibodies. Continued or repeated antigenic stimulation leads to differentiation of the activated B cell into a plasma cell, which secretes copious amounts of immunoglobulins. These carry the same antigenic specificity as the original B cell, but are usually of the IgG type. The latter phenomenon is called isotype switching and depends on T cell signals (T cell help).

T lymphocytes are differentiated in the thymus into two major subclasses, CD8+ and CD4+, that may be distinguished by the presence of the respective glycoproteins. CD8 serves as a co-receptor molecule for major histocompatibility complex (MHC) class I molecules, while CD4 serves as a co-receptor molecule of MHC class II molecules. The activation of T cells depends not only on the mere presence of its cognate antigen but on its binding, in fragmented form, to MHC proteins on antigen-presenting cells.

MHC class I dependent antigens are synthesized by antigen-presenting cells. Peptide fragments of a nascent protein antigen associate with MHC class I proteins in the Golgi compartment. The peptide-MHC-I complex is transported to the cell surface, where it can be recognized by T cells that simultaneously carry CD8 molecules that ligate the MHC class I protein on the antigen-presenting cell and

T cell antigen receptors (TCRs) that can recognize the peptide. The MHC-I pathway is therefore an endosomal pathway for antigen presentation. Among MHC class I dependent antigens are viral antigens produced in a virus-infected cell. CD8+ cells recognize these antigens and lyse the antigen-expressing cell by a machinery containing several cytotoxic and pro-apoptotic factors.

CD4+ cells recognize antigens presented in the presence of MHC class II proteins. This characteristically occurs in specialized antigen-presenting cells (APCs) of the macrophage lineage. Such APCs internalize antigens by endocytosis, which is followed by lysosomal digestion. Antigenic peptide fragments escape after partial proteolysis in pro-lysosomal compartments, associate with MHC-II molecules, and appear on the cell surface where they are available for recognition by CD4+ cells. Specialized APCs, called dendritic cells, patrol peripheral tissues including blood vessel walls, where they internalize large numbers of antigens, process them for MHC class II dependent presentation and recirculate to lymph nodes for delivery to specific T cells. Upon cytokine stimulation, dendritic cells 'freeze' their peptide-MHC-II complexes in high density on their surfaces, which leads to efficient activation of antigen-specific T cells.

Undifferentiated 'naive' precursor CD4+ T cells proliferate upon primary activation and produce interleukin-2 (IL-2), but have a limited effector spectrum. Upon reactivation, they differentiate into Th1 or Th2 effector cells, depending on the cytokine signals received by the cell during activation. As an initial step in this pathway, the reactivated T cells may express a large variety of cytokines and are referred to as Th0 cells. Th1 differentiation is promoted by the cytokine IL-12 that is produced by macrophages and NK cells in inflammatory lesions and lymphoid tissues. Th1 cells are considered to be pro-inflammatory cells that function to activate macrophages and activate the defence against intracellular microorganisms. The major cytokine secretions on activation of Th1 cells are IL-2, interferon- γ and tumour necrosis factors (both TNF- α and TNF- β , also termed lymphotoxin).

Conversely, Th2 cells are considered to be anti-inflammatory and have a major function in assisting in the activation of B lymphocytes to generate antibodies. Their development is stimulated by IL-4 produced by surrounding T cells, eosinophils, and mast cells. Activated Th2 cells secrete a myriad of cytokines including IL-4, -5, -6, -10, and -13. The overall effect of these activities is to stimulate allergic responses and the defence against parasites and extracellular microorganisms.

A third type of Th cell, Th3, has recently been described. Its major secretory product is transforming growth factor- β (TGF- β) that also promotes its development in an autocrine manner. Th3 cell differentiation characteristically occurs among mucosal lymphocytes and after oral immunization. Since TGF- β is anti-inflammatory and inhibits activation of T cells and macrophages, it is possible that TGF- β -secreting Th3 cells account for the suppressor cell activity that is often observed in parallel with immune activation.

All T cells depend on immunoglobulin-like antigen receptors (TCRs) for recognition of antigen-MHC complexes. TCRs develop through somatic rearrangement during T blast differentiation in a process that is analogous to the immunoglobulin gene

rearrangement during B blast development. Among many different variable (V) gene segments present in the genome, one is randomly selected in each cell and fused to diversity (D), joining (J) and constant (C) segments. Together, these V-D-J-C gene segments form a functioning TCR gene that can be transcribed and translated. An α and a β chain gene product formed by this mechanism associate to form the dimeric TCR, which contains complementarity-determining regions used for binding of antigenic peptides. The conformation and, therefore, the sequence of the TCR determines the specificity of the T cell.

Only a small proportion of all TCRs formed by the stochastic process of somatic rearrangement is used for antigen recognition in the adult individual. This is due to the process of clonal selection, which first eliminates all T cells carrying TCRs that are unable to bind MHC. In a second step, T cells that express TCRs that bind MHC-self-antigen complexes with high affinity are killed. This leaves approximately 1–2% of all T cells surviving and able to leave the thymus to fight antigens throughout the body. Such T cells have the capacity to bind self-MHC molecules that have bound 'foreign' antigenic peptides. In theory, all autoreactive T cells should have been eliminated in the thymus.

More than 95% of all T cells use TCR $\alpha\beta$ antigen receptors, recognize MHC-peptide complexes, and are selected through thymic education. However, a small proportion of T cells express another type of TCR, a $\gamma\delta$ dimer that is structurally related to, but functionally different from, TCR $\alpha\beta$. $\gamma\delta$ T cells do not interact with MHC proteins. Instead, they bind to CD1 proteins expressed on the surface of antigen-presenting cells. Importantly, antigens bound to CD1 are often complex lipids rather than oligopeptides, $\gamma\delta$ T cells do not undergo thymic education but appear to mature in the bone marrow and mucosal tissues. In addition to $\gamma\delta$ T cells, certain $\alpha\beta$ T cells are also MHC-independent and CD1-restricted. These include the NK-T cells that express a few specific types of TCR $\alpha\beta$ molecules that recognize antigen-CD1 complexes and exhibit cytotoxic NK-like activity upon activation.

To summarize, the effector mechanisms of adaptive immunity include antibodies, cytotoxic activity, and cytokines regulating immunity and inflammation. All these actions are initiated when antigen receptors on B and T cells encounter their cognate antigens. In order to evoke immune effector responses, initial antigen recognition has to be followed by a series of events involving cytokines, cell surface receptors, and signalling pathways on interacting T cells, B cells, macrophages and other immune cells. The precise type and order of such interactions determine the effector mechanism and, hence, the type of immune response that is mounted upon each encounter of antigen.

11.3 Effect of humoral immunity on atherogenesis

Deposits of immunoglobulins have been detected in human atherosclerotic lesions.⁽²⁾ These antibodies may largely be derived from the circulating blood, but some of them could emerge from B cells and plasma cells of lesions and the

periadventitial connective tissue.⁽³⁾ An implication that this accumulation of immunoglobulins is related to the disease process is derived from the presence of activated complement complexes in atherosclerotic lesions. The classic pathway of complement activation is initiated by binding of antibody to the C1 component of this multiprotein system. This initiates a cascade that results in the formation of a C5b-9 terminal complex. This end product of the cascade has been detected in atherosclerotic lesions from both humans⁽⁴⁾ and animals.⁽⁵⁾ Although it does not cause a massive lytic response in lesions, it may provoke many other atherogenic responses, such as stimulating the secretion of monocyte chemoattractant protein-1 (MCP-1) and IL-8.⁽⁶⁾ A causal role of complement in atherogenesis has been implicated by the marked reduction in extent of lesions formed in complement C6 deficiency cholesterol-fed rabbits.⁽⁷⁾

A major focus of humoral immunity is on antibodies to oxidized forms of LDL. IgG that recognizes both malondialdehyde and copper modified forms of LDL has been detected in atherosclerotic lesions.⁽⁸⁾ Furthermore, titres of IgG autoantibodies to oxidized LDL have also been detected in serum. These titres have been implicated as a diagnostic tool that is positively correlated to the severity of human atherosclerosis in both the carotid and coronary arteries.^(9, 10) However, a lack of correlations has also been reported.⁽¹¹⁾ Part of the disagreement between studies may be related to the lack of standardization of assays for autoantibodies to oxidized lipoproteins.⁽¹²⁾ The inconsistencies may also be in part due to the complexity of oxidized forms of LDL which may contain a variable amount of many antigenic lipid and protein moieties.

To determine whether autoantibodies against oxidized LDL are causal in atherogenesis, titres have been increased in Watanabe heritable hyperlipidemic rabbits,⁽¹³⁾ cholesterol-fed rabbits,⁽¹⁴⁾ and LDL receptor-deficient mice⁽¹⁵⁾ by immunization. Although the earlier human studies showed that autoantibody titres were positively correlated with the severity of atherosclerosis, each of these studies demonstrated that increased titres lead to decreased severity of disease. Overall, these studies demonstrate that humoral responses to modified forms of LDL may have profound effects on the atherogenic process, although this is a complex interaction that needs to be defined further.

11.4 Presence of T lymphocytes in atherosclerotic lesions

11.4.1 Studies in human tissues

Substantial numbers of T lymphocytes have been detected in specific regions of human atherosclerotic lesions.⁽¹⁶⁾ T lymphocytes may precede the entry of mononuclear cells in the earliest stages of lesion formation. Furthermore, their numbers may be greater than that of macrophages.^(17, 18) As lesions progress, T lymphocytes have a distinct regional distribution (Fig. 11.2). They are present in numbers comparable to

the numbers of macrophages in the shoulder and fibrous cap regions of more advanced lesions, making up approximately 20% of the total cell population. T lymphocytes represent a lesser number of the cells in the lipid core, but still account for nearly 10%.⁽¹⁶⁾ Sites of coronary thrombosis, whether caused by erosion or rupture, are closely associated with lesion regions that are composed of activated T lymphocytes.⁽¹⁹⁾ There is a potential for these cells to be recruited and activated as a consequence of the thrombotic event, although the location of this cell type within lesions is consistent with their presence prior to the thrombotic event. Thus, a role of T lymphocytes in atherogenesis may be inferred by their presence in lesions from the initiation phase to the final stage that causes acute clinical events.

Several investigators have reported on the phenotype of T lymphocytes within atherosclerotic lesions. CD4/CD8 ratios vary widely between studies, but most investigators find a predominance of CD4+ cells in advanced lesions.^(16, 20, 21) Resident lymphocytes have also been characterized by their expression of TCR $\alpha\beta$ and TCR $\gamma\delta$ antigen receptors. In one study a predominance of $\alpha\beta$ chains was demonstrated, as would be expected since ~95% of peripheral lymphocytes in blood express this form of the T cell receptor.⁽²²⁾ However, a relative enrichment of $\gamma\delta$ T cells in lesions compared with blood has been reported.⁽²³⁾ It remains to be determined whether such cells recognize lipid antigens derived from the plaque.

The mere presence of T lymphocytes does not necessarily imply that these cells are performing an adaptive immune response, since lymphocytes could be attracted to lesions by the combination of adhesion molecules and chemoattractants that are

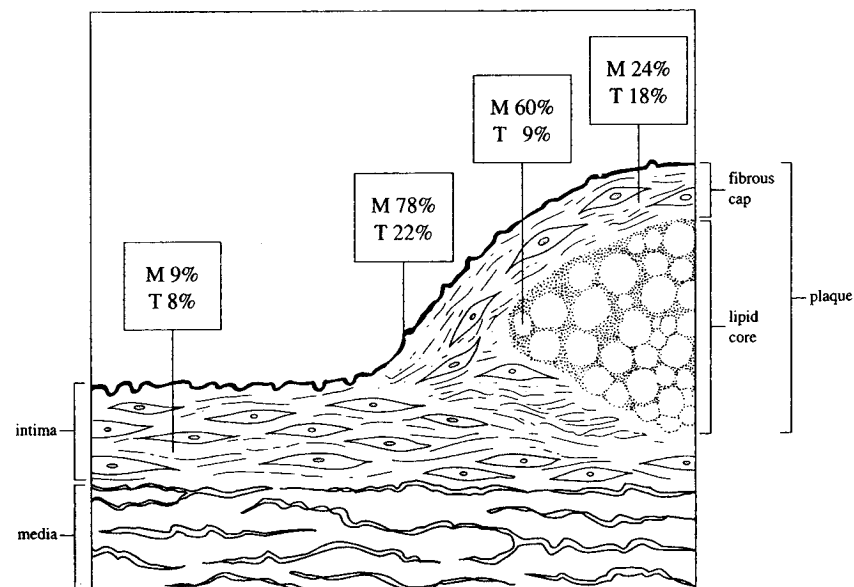


Fig. 11.2 The distribution of T lymphocytes and monocyte-derived macrophages in specific regions of advanced human atherosclerotic lesions.

expressed in diseased tissue. For example, the combined expression of vascular cell adhesion molecule-1 (VCAM-1) and MCP-1 would be sufficient to explain the presence of lymphocytes in lesions. To determine whether T lymphocytes are involved in an immunological response, immunocytochemical analysis has been performed to detect activation markers. The activation markers MHC class II, very late activation antigen-1, and the cytokine, interferon- γ , are present on many cells. Interleukin-2 receptors (CD25) are also present on a smaller number of cells.^(24, 25) Consistent with the presence of CD25, T lymphocyte proliferation occurs in human lesions.⁽²⁶⁾ Additional evidence that T lymphocytes are activated within atherosclerotic lesions is implied by the close association of this cell type with macrophages. T lymphocytes are frequently directly apposed to macrophages in lesions and have been shown to be linked by specialized membrane contacts that are consistent with a functional interaction.^(27, 28)

A further method of determining whether T cells are activated by specific mechanisms within lesions is to define potential antigens present in atherosclerosis. The gene reorganization that provides the basis of the specificity of TCRs enables determination of whether a population of cells is mono- or polyclonal. Studies of advanced human lesions have been consistent with heterogeneous TCR gene rearrangement, thus indicative of a polyclonal population.^(22, 28) However, early lesions in an experimental murine model show a restricted TCR $\alpha\beta$ heterogeneity and the presence of oligoclonal T cells.⁽²⁹⁾ This points to activation and clonal expansion of a small set of T cells that probably recognize specific antigens of the lesions. However, as mentioned previously, one of the major proposed antigenic particles, oxidized LDL, has the potential to contain many antigenic moieties. Therefore, this one complex entity could lead to a polyclonal response.

T cell activation has also been inferred from the presence of activation markers on many cell types present in lesions. A distinguishing property of interferon- γ in this respect is its ability to stimulate the expression of MHC class II on smooth muscle and endothelial cells. Therefore, the consistent finding of MHC class II expression on these cell types in human atherosclerotic lesions implies the secretion of interferon- γ .^(16, 30) In addition to activation of macrophages, interferon- γ activates endothelial cells to express adhesion molecules and pro-thrombotic surface molecules, and it reduces proliferation, contractility, α -actin expression, and collagen production by smooth muscle cells. All these effects likely contribute to inflammation in the formation of atherosclerotic lesions.

To determine a mechanism of activation, one approach has been to establish T lymphocyte clones from human atherosclerotic lesions and expose these to candidate antigens. One candidate antigen is oxidized LDL that is present in atherosclerotic lesions.^(31, 32) About 15% of CD4+ clones established from human atherosclerotic lesions responded to the presentation of oxidized LDL by proliferation and secretion of cytokines.⁽³³⁾ This response required the presence of autologous antigen-presenting cells, which is consistent with the presence of committed CD4+ cells. In the presence of both antigen and antigen-presenting cells, all the positive clones responded to

oxidized LDL with secretion of interferon- γ , while only 25% secreted interleukin-4. In contrast, oxidized LDL was unable to generate a proliferative response to any clones generated from peripheral blood. Therefore, at least part of the activation of T lymphocytes in lesions is attributable to the presence of oxidized LDL. Similar data are also available that a population of T lymphocytes in rabbit lesions will proliferate in response to heat shock protein 65.⁽³⁴⁾ Currently, oxidized LDL and heat shock protein 60/65 are on a short list of potential antigens that are thought to promote T lymphocyte responses within atherosclerotic lesions.

Other emerging antigens include those derived from viruses and bacteria.⁽³⁵⁾ Viruses of the herpes family have been detected in atherosclerotic lesions by several investigators. Cytomegalovirus (a member of the herpes family) has been linked to transplant arteriosclerosis, and Marek's disease virus (another herpes virus) accelerates arteriosclerosis in cholesterol-fed chickens. Finally, *Chlamydia pneumoniae* has been linked to atherosclerosis in seroepidemiological studies and has been isolated from human atherosclerotic tissue.⁽³⁶⁾ It remains to be determined to what extent the proposed pro-atherogenic effects of these microbes are due to immune responses or to direct effects of the microorganisms on the vascular cells.

Further insight on lymphocyte properties within lesions may be gleaned from the spectrum of cytokines that have been secreted in the diseased tissue. As noted earlier, activation of lymphocytes can lead to an array of cytokines being secreted, and the type of cytokine secreted can provide an insight into the differentiation status of the cell. Two of the principal cytokines that have been studied are Interferon- γ and interleukin-4, as indicative of the presence of Th1 and Th2 cells, respectively. Interferon- γ has been the most consistently detected cytokine, both at the protein and mRNA levels.^(24, 37) However, NK cells can also secrete substantial interferon- γ , although there is currently no indication that this cell type is present in atherosclerotic lesions. Finally, recent data suggest that macrophages may secrete interferon- γ under certain circumstances.⁽³⁸⁾ Interleukin-4 has been detected in only a small number of lesions.^(39, 40) Again, any detection of interleukin-4 would have to take into account its secretion by other cell types, especially mast cells that have been detected in human lesions.⁽⁴¹⁾ In conclusion, while many questions remain, currently available data on human atherosclerotic plaques point to a predominance of Th1 cytokines. This would suggest that cellular immune responses in lesions promote a macrophage-activating inflammation resembling delayed-type hypersensitivity reactions.

The ability to detect cytokines, either at the mRNA or protein level, is difficult in human atherosclerotic lesions. Acquisition of human vascular tissue in a fresh state to permit isolation of mRNA is only permissible under specific conditions such as carotid endarterectomy. However, only lesions that are in advanced disease stages can be obtained by such procedures. There are insurmountable barriers to the routine acquisition of early or intermediate lesions from humans to permit reliable detection of mRNA or protein. This problem is compounded for lymphocyte-derived cytokines, since they are usually secreted in small discrete areas termed

immunological synapses. In a chronic inflammatory response such as atherosclerosis, cytokines exert powerful actions when secreted in masses that may not be detectable by currently available technology. Therefore, while positive results implicate a specific lymphocyte reaction, negative results do not negate the involvement of a specific cytokine in the human disease process.

11.4.2 Studies in animal tissues

Much of our information on the cellular sequence of events in atherogenesis has been derived from animal models of the disease. While few of these studies have included lymphocytes, the inclusion of immunocytochemical procedures to quantify and characterize this cell type is becoming increasingly common. One model that has been used in a large number of studies is rabbits fed a cholesterol-enriched diet. Despite this being a simple dietary stimulus to the formation of atherosclerotic lesions, lymphocytes are present in lesions from these animals, albeit in relatively small numbers.⁽⁴²⁻⁴⁴⁾

Contemporary atherosclerosis studies are more commonly performed in mice. Early atherosclerosis studies used C57BL/6 mice fed a diet enriched in cholesterol, cholate, and saturated fat. These mice develop small lesions that are restricted to the aortic root. The lesions have a simple morphology of macrophages and no T lymphocytes. There is now an increased use of mice that have been genetically manipulated to generate pronounced lesions either spontaneously or in response to modified diets. The most commonly used are LDL receptor $-/-$ and apoE $-/-$, both of which develop lesions in many regions of the vasculature. Lesions in LDL receptor $-/-$ mice are composed predominantly of lipid-laden macrophages, while those present in apoE $-/-$ mice progress to a more complex morphology.⁽⁴⁵⁻⁴⁷⁾ Lesions from both these strains contain T lymphocytes, which are predominantly CD4+.⁽⁴⁸⁻⁵⁰⁾ In lesions from apoE $-/-$ mice, these cells are clustered in either the luminal aspect of lesions,^(48, 51) or in other regions consistent with a local proliferative response.⁽⁴⁹⁾ Studies in both mice⁽⁴⁸⁾ and rats⁽⁵²⁾ have demonstrated that the presence of T lymphocytes relative to macrophages is greatest at the earlier stages of the disease evolution.

While there is solid evidence for the presence of T lymphocytes at all stages of the atherogenic process, B lymphocytes have not been consistently detected, despite the large amounts of immunoglobulins present within lesions. However, expressions of immunoglobulin genes have been detected by differential hybridization techniques in lesions from Watanabe heritable hyperlipidemic rabbits. This study also performed electron microscopic analysis of lesions and was able to demonstrate the presence of plasma cells.⁽⁵³⁾ B lymphocytes have also been observed in lesions from apoE-deficient mice by immunocytochemical detection of CD22.⁽⁵⁴⁾

There have been more limited studies to define the presence of cytokines in lesions from animal models. However, the limited information derived from lesions of apoE $-/-$ mice is consistent with findings in human tissue, with interferon- γ

being present in all atherosclerotic arteries that were examined. Conversely, interleukin-4 was only detected in lesions of apoE $-/-$ that were fed a modified diet that provoked a severely hyperlipidemic response.⁽⁵⁵⁾

Overall, there is abundant evidence that lymphocytes are present in the atherosclerotic lesions of many animal models of atherosclerosis and particularly in genetically manipulated forms of mice. Therefore, this infers that regulation of lymphocyte function in these models should modulate formation of atherosclerotic lesions if this cell type has an active role in the disease process.

11.5 Effects of regulation of T lymphocyte function on atherogenesis

The presence of activated lymphocytes at each stage of the human lesion formation provides compelling evidence for a role of this cell type in the disease process. However, defining the nature of that role will provide a formidable obstacle. There are limited circumstances in humans in which lymphocyte function is modified so that the effect on atherosclerosis can be demonstrated as a secondary endpoint.

Some potential insight into the role of lymphocytes in the development of atherosclerosis in humans can be assimilated from individuals who are engaged in a course of immunosuppressive therapy. These are predominantly transplant groups of patients. Unfortunately, these patients are seldom on monotherapy, and there is the confounding variable of the presence of the transplanted organ. In the case of heart transplants, the donated organ is well known to have greatly increased vascular disease. However, the pathology of this disease is not the same as atherosclerotic lesions present in non-transplant tissues. Some information could be obtained by quantifying and characterizing atherosclerosis in non-transplanted areas of immunosuppressed patients, although no systematic study has been performed.

Individuals with AIDS may also provide an understanding into the role of lymphocytes in atherosclerosis. Indeed, there are some indications that patients with AIDS have markedly increased severity of atherosclerosis.⁽⁵⁶⁾ While prolonging the interval between HIV infection and clinical AIDS, the recently introduced HAART combination therapy may lead to increased cardiovascular disease. However, the complexity of the disease in HIV-infected individuals, the frequent occurrence of associated complications, and the fact that current therapy elevates risk factors for atherosclerosis provides a barrier to clear extrapolation of the specific effects of immune suppression on atherogenesis.

Given the barriers to obtaining information on immunosuppression in humans, definition of the role of lymphocytes in atherogenesis will have to be garnered from animal experimentation. There have been multiple ways of addressing this problem which

generally involves the inhibition of lymphocyte function. Discussed below are the results obtained from several different modes of manipulating the immune system.

11.5.1 Antibody ablation approaches

T lymphocyte subpopulations can be markedly ablated by administration of antibodies to specific cell markers. The first experiments were performed in a rat model of vascular injury.⁽⁵⁷⁾ T cell ablation with a cytolytic antibody increased restenosis lesions, implying an inhibitory role in the proliferative response of smooth muscle cells. However, the post-injury lesion lacks a significant inflammatory component and therefore differs from the early atherosclerotic lesion that is dominated by inflammatory cells.

When antibody ablation was used to decrease T cells in C57BL/6 mice fed a high-fat diet,⁽⁵⁸⁾ ablation of CD4+ cells dramatically reduced the formation of atherosclerosis in the aortic root. CD8+ cell ablation also produced a modest, but statistically significant, decrease in atherosclerosis. Furthermore, this effect could have been related to this treatment promoting a decrease in plasma cholesterol by an undefined mechanism. The combined ablation of CD4+ and CD8+ cells did not have an additive effect. The magnitude of this effect of ablation is surprising given the lack of T lymphocytes that have been demonstrated in this model.

Deletion of specific lymphocyte populations with antibodies has not been performed in genetically altered mice that exhibit larger and more complex lesions. However, a recent study has used this approach to inhibit CD40 signalling in LDL receptor-deficient mice. Signalling through this molecule is related both to humoral and cellular adaptive immunity following interaction with the CD40 ligand. The CD40 ligand is a non-soluble effector molecule that reacts with CD40 that is present on many of the cell types present in lesions. In support of a role of this molecule in atherosclerosis, anti-CD40 ligand antibody administration into LDL receptor-deficient mice reduced both the size and lipid content of lesions.⁽⁵⁹⁾ This ablation was also associated with a decreased presence of macrophages and T lymphocytes in lesions. The CD40 ligand was previously considered to be specific for lymphocytes, although the functional molecule has recently been detected on endothelial cells, smooth muscle cells, and macrophages.⁽⁶⁰⁾ Therefore, at present the inhibition of the CD40 ligand cannot be taken as definitive evidence of the role of T lymphocytes.

An alternative approach is to modulate immune activity by administration of large doses of polyclonal immunoglobulins. In addition to transfer of specific antibodies, this can lead to inhibition of cellular immune responses. Such therapy is used successfully to prevent coronary inflammatory disease and myocardial infarction in children with Kawasaki disease. Nicoletti *et al.*⁽⁶¹⁾ found that a few injections of polyclonal immunoglobulins reduced atherosclerotic lesions in apoE-deficient mice by ~50%. This supports the notion that atherosclerosis is an inflammatory disease that can be controlled by immunotherapy.

11.5.2 Pharmacological approaches

There has been a relatively limited number of studies in which conventional pharmacological agents have been used to suppress the adaptive immune response, which in part is due to the limited armory of immunosuppressive drugs. Cyclosporin A has been the most widely used drug for immunosuppressive studies in atherosclerosis. The first study was performed in C57BL/6 mice fed a diet enriched in cholesterol, cholate, and saturated fats. While the administration of cyclosporin A was associated with an increase in the extent of atherosclerosis in the aortic root, there was also a profound increase in plasma cholesterol concentrations that negates definition of a mechanism based specifically on lymphocyte inhibition.⁽⁶²⁾ Two further studies have determined the effect of cyclosporin A on the development of atherosclerotic lesions in cholesterol-fed rabbits. However, conflicting results were obtained with the drug either inhibiting⁽⁶³⁾ or promoting⁽⁴²⁾ the extent of the disease. Cyclosporin A had no effect on plasma lipid concentrations in either study. Roselaar *et al.*⁽⁴²⁾ administered cyclosporin A initially in a daily loading dose followed by a maintenance dose to titre blood concentrations between 100 and 200 ng ml⁻¹. This scheme provided effective immunosuppression as defined by the maintenance of patency of an allogeneic skin graft. Conversely, Drew *et al.*⁽⁶³⁾ administered cyclosporin A on a daily basis that led to blood concentrations at the terminal bleed of over 600 mg ml⁻¹. Despite these high blood concentrations, there was no demonstration of overt toxicity, as defined by plasma creatinine concentrations. The difference in the results may be related to the spectrum of activities that cyclosporin A can exert on lymphocyte function. Smaller doses are more selective on Th1 T lymphocyte subtypes, while higher doses act on a broader spectrum of lymphocyte subtypes and may also inhibit smooth muscle proliferation.⁽⁶⁴⁾

A more recent study has administered FK506 to cholesterol-fed rabbits.⁽⁶⁶⁾ FK506 is a newer immunosuppressive agent that is structurally distinct from cyclosporin A. It has a similar pharmacological profile in reducing the production of the secretion of IL-2, IL-3, and interferon- γ , but with much greater efficacy.⁽⁶⁶⁾ FK506 produced a dose-dependent increase in the extent of atherosclerotic lesions covering the aortic intima. The mechanism of this effect of FK506 was attributed to the promotion of cholesterol esterification in macrophages.

While pharmacological approaches have provided an appreciation that lymphocytes have a role in the atherogenic process, the limited number of drugs have poorly defined mechanisms of action. Therefore, there is limited usefulness of this approach for providing mechanistic insight into defining a specific function of lymphocytes in the atherogenic process.

11.5.3 Genetic approaches

Probably the most useful approach to define mechanisms by which lymphocytes influence the atherogenic process is the use of mice with specific genetic manipulations of the immune system.

Initial investigations used mice strains in which the development of atherosclerosis required the feeding of a diet enriched in saturated fat, cholate, and cholesterol. One of the earliest studies was unable to demonstrate changes in the severity of aortic root lesion formation in mice with severe combined immune deficiency, athymic nude mice, or MHC class II deficiency.⁽⁶⁷⁾ However, since T lymphocytes have not been detected in lesions of this model, it is unclear that this is a suitable model to study the effects of lymphocyte deficiency. Despite this lack of appropriate cellularity, deficiency of MHC class I led to a three-fold increase in lesion size. A subsequent study also quantified the development of atherosclerosis in nude (nu/nu) C57BL/6 mice fed a modified diet.⁽⁵⁸⁾ In this study, there was a pronounced decrease in atherosclerosis in the aortic root with lesion size in homozygous nude (nu/nu) mice, being only 10% of that obtained in the heterozygotes (nu/+). The reason for the disparity between these studies is not apparent. In agreement with the study of Emeson *et al.*,⁽⁵⁸⁾ a further study was performed in rats that had the same nude phenotype that demonstrated euthymic (rnu/+) rats developed larger lesions than rnu/rnu rats. Furthermore, these lesions contained a greater number of macrophages that were more engorged with lipid.⁽⁶⁸⁾ In contrast, genetic T cell deficiency (mu/rnu) led to enhanced proliferative post-injury lesions in rats.⁽⁵⁷⁾ The discrepancy in results between smooth muscle-dominated post-injury lesions and macrophage-dominated fatty streak lesions clearly indicates that the mechanisms driving the growth of these two different pathologies are completely different.

More recent studies have used compound genetically manipulated mice in a specific immune deficiency bred into mice of an atherosclerosis-susceptible background. The most commonly used of these atherosclerosis-susceptible mice are the apoE-deficient strain.^(69, 70) Unlike the lesions formed in C57BL/6 mice, several studies have demonstrated the presence of T lymphocytes in lesions of these mice.^(48–50)

Two studies have compared the effects of total lymphocyte deficiency in the development of lesions in apoE $-/-$ mice.^(51, 71) These studies used mice that were deficient in genes of the recombinant activation gene cascade. This deficiency leads to an inability to perform VDJ rearrangement, thus negating the ability to produce mature B or T lymphocytes. In both studies, there was no effect of total lymphocyte deficiency on the development of atherosclerosis in mice fed a diet enriched in saturated fat that promoted a dramatic hypercholesterolemic response (with serum cholesterol concentrations of approximately $1000\text{--}2000\text{ mg dl}^{-1}$, or $25\text{--}50\text{ mmol l}^{-1}$). In contrast, mice maintained on a normal laboratory diet had a 43% reduction in the severity of atherosclerotic lesions in the aortic root. Therefore, these studies indicate that lymphocytes modulate atherogenesis in apoE $-/-$ mice but this regulatory effect is overwhelmed by excessive hypercholesterolemia.

Several recent studies have defined the effects of a specific cytokine or cell surface molecule on atherogenesis. One example in the lymphocyte field is interferon- γ receptor-deficient animals that were bred into an apoE-deficient background.⁽⁵⁰⁾ Interferon- γ receptor-deficient mice had a decrease in atherosclerosis, thus providing evidence for a pro-atherogenic role of interferon- γ . In addition to quantitative changes,

lesions also had decreased lipid deposition, reduced cellularity, and increased collagen content. This effect may not have been accounted for completely by local immune reactions within the vessel wall, since compound deficient mice also had an increase in potentially protective phospholipid/apoA-IV particles. Furthermore, this study is potentially compromised by some variation in the genetic background of the single, compared to the compound, deficient mice. Nevertheless, it provides the first indication that a specific lymphocyte-derived cytokine may influence the atherogenic process.

The importance of CD40 signalling was recently confirmed when CD40 ligand (CD154)-deficient mice were crossed with apoE-deficient animals.⁽⁷²⁾ The compound mutant mice showed a significant reduction in atherosclerotic lesions compared to apoE single-knockout mice. Interestingly, the effect of CD40 signalling could be ascribed to a role in the progression rather than the initiation of lesions.

It is likely that there will be an increasing number of studies performed on compound deficient animals. The specificity of the immune deficiency that is produced by genetic manipulation is a distinct advantage of this approach. However, this approach may also highlight some of the contradictions that are likely to arise in this field. Different mouse strains have long been known to provide differing responses to stimuli that activate adaptive immune responses. For example, the commonly used strain in atherosclerotic studies, C57BL/6, is known to be more oriented toward Th1 responses, while BALB/c mice are more oriented toward Th2 responses. Therefore, in addition to the many potentially confounding factors in quantifying atherosclerosis in mice (reviewed in Ref. (73)) careful attention must be applied to strain in data interpretation.

11.6 Potential effects of lymphocyte-derived cytokines on atherogenesis

Activated lymphocytes secrete a considerable repertoire of cytokines that have the potential to influence every stage of the atherogenic process by an influence on all the cell types involved in the disease process. Some of these effects are highlighted in Table 11.1, which shows there is the potential for lymphocyte-derived cytokines to exert a wide array of mechanisms. The table focuses on selected effects of interferon- γ and interleukin-4 and -13 as examples of the cytokines that define the distinction between Th1 or Th2 lymphocyte subpopulations. This illustrates that many of these potential effects of lymphocyte-derived cytokines can be influenced by antagonistic mechanisms of these selected cytokines. Therefore, this emphasizes the need to determine the specific nature of the lymphocyte populations that infiltrate lesions.

11.7 Conclusions

An important role of lymphocytes in the development of atherosclerosis is heavily implied by the large number of activated lymphocytes that are present at all stages

Table 11.1 Selected effects of cytokines released from activated lymphocytes on the mechanisms that have been implicated in the atherogenic process

Potential mechanism influencing atherogenesis	Cytokines that increase the process	Cytokines that decrease the process
LDL oxidation		Interferon- γ ⁽⁷⁴⁾
15-Lipoxygenase expression	Interleukin-4 ⁽⁷⁵⁾	Interferon- γ ⁽⁷⁵⁾
	Interleukin-13 ⁽⁷⁶⁾	
Class A scavenger receptor expression	Interleukin-4 ⁽⁷⁷⁾	Interferon- γ ⁽⁷⁸⁾
CD36 expression	Interleukin-4 ⁽⁷⁹⁾	
Nitric oxide production	Interferon- γ ⁽⁸⁰⁾	Interleukin-4 ⁽⁸¹⁾ Interleukin-13 ⁽⁸¹⁾
VCAM-1 expression	Interleukin-4 ⁽⁸²⁾	
Smooth muscle cell growth		Interferon- γ ⁽⁸³⁾

of lesion development. Based on these data, it would seem that lymphocytes are an integral component of the disease process and that adaptive immunity to either self or foreign antigens is important in the pathogenesis of atherosclerosis. At the present time, the complexities of both the atherogenic process and the lymphocytic system have confounded attempts to define the specific role of adaptive immunity in the development of lesions. However, the increased availability of mice with specific immune deficiencies is likely to lead to a great understanding of the experimental disease process that will hopefully be extrapolated to the human disease.

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