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Lymphocytes and the Adventitial Immune Response in Atherosclerosis

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Abstract

Though much of the research on atherosclerosis has focused on the intimal accumulation of lipids and inflammatory cells, there is an increasing amount of interest in the role of the adventitia in coordinating the immune response in atherosclerosis. In this review of the contributions of the adventitia and adventitial lymphocytes to the development of atherosclerosis, we discuss recent research on the formation and structural nature of adventitial immune aggregates, potential mechanisms of crosstalk between the intima, media, and adventitia, specific contributions of B lymphocytes and T lymphocytes, and the role of the vasa vasorum and surrounding perivascular adipose tissue (PVAT). Furthermore, we highlight techniques for the imaging of lymphocytes in the vasculature.

Keywords

Lymphocytes; Atherosclerosis; Adventitia; Imaging

Introduction

Atherosclerosis and its clinical manifestations of stroke and myocardial infarction are the leading cause of morbidity and mortality in the Western world[1]. Understanding the pathophysiology of atherosclerosis and potential means of preventing its progression are of critical importance since the initial manifestation of coronary artery disease is sudden cardiac death or myocardial infarction in over half of individuals[2]. Though much emphasis has been placed on investigating atherosclerosis through intimal accumulation of lipids and inflammatory cells, recent research suggests the adventitia may also play a critical role in coordinating the progression of the disease. It has long been known that damage to the

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adventitia in the setting of both percutaneous coronary angioplasty[3,4] and the placement of a circumferential silastic collar[5] can precede neointimal formation in porcine and rabbit models, and more recent studies have elucidated some of the potential mechanisms of this adventitial contribution to lesion development. The adventitia is a major site of lymphocyte accumulation and organization[6–8], and histological studies in both humans and mice suggest that the adventitia may contribute to the adaptive and innate immune responses that regulate atherosclerosis[6,7,9–11]. These studies further underscore the current notion that immunomodulatory therapy may be an effective strategy to add to our clinical arsenal for the treatment of patients[12]. Understanding how the surrounding tissues such as the perivascular fat and the vasa vasorum influence lymphocyte recruitment and subsequent modulation of atherosclerosis progression may ultimately elucidate mechanisms of crosstalk between adventitial lymphocytes and the atherosclerotic plaque.

Molecular and cellular imaging can serve as a powerful tool to aid our understanding of the pathophysiology of atherosclerosis[13]. Given the close proximity of the different layers of the arterial wall, careful selection of the imaging target and imaging modality is critical. Targeted molecular imaging of intimal macrophage density could provide a means to not only determine the degree of inflammation in atherosclerotic plaque[14–16], but also monitor response to medical therapy[17]. Advances in targeted cellular and molecular imaging make it possible not only to identify the presence of lymphocytes within the adventitia and atherosclerotic plaque but to also study the role lymphocytes play in the progression of atherosclerosis. However, differentiation between cells within the intima and adventitia using certain imaging modalities may prove very challenging. Thus, the use of multimodality imaging may provide adequate sensitivity and spatial resolution to reliably localize labeled cells or molecular targets within the arterial wall and provide functional information on their role in atherosclerosis progression.

Immune cells in the Adventitia of Diseased Arteries

The presence of lymphocytes in the adventitia surrounding atherosclerotic lesions has long been recognized[8], yet our understanding of their organization and their role in the progression of atherosclerosis is just recently unfolding. The majority of murine work in this area uses the aorta as a model as the small size of murine coronary arteries make detailed study of their structure and function challenging. The aggregation of lymphocytes and formation of organized structures critically depend on surrounding adventitial cells that orchestrate this process. Murine studies have demonstrated the presence of leukocytes in the adventitia adjacent to atherosclerotic lesions that are comprised of collections of T lymphocytes, B lymphocytes, follicular dendritic cells and tingible body macrophages (predominantly found in germinal centers)[6,7,10,18,19].

Structural organization of the adventitial infiltrates has been shown to range from loose T lymphocyte aggregates to lymphoid follicles with B lymphocyte and dendritic cell nodular centers surrounded by parafollicular T lymphocytes, termed aortic tertiary lymphoid organs (ATLOs)[6,7,10,18–20]. ATLOs can be distinguished from paraaortic lymph nodes as ATLOs border the external elastic lamina and lack capsules[10]. The presence of these ATLOs in the adventitia has been associated with advanced atherosclerotic plaques[6–8,10], suggesting that they may act as centers of a local humoral response with B lymphocyte subset selection, maturation, and antibody production following antigen presentation by dendritic cells[6,7].

Several structural aspects of these advanced adventitial lymphoid aggregates support this idea. Lymphoid aggregates in the adventitia of a patient with chronic periaortitis were found to react positively to peanut lectin, which binds B cells in germinal centers[19]. Consistent with this idea, recent important work by Grabner and colleagues, demonstrated that

adventitial B cell follicles ($B220^+$) contained follicular dendritic cell networks and T cell ($CD3\varepsilon^+$) and plasma cell ($CD138^+$) compartments, an organization that promotes antigen presentation, selection, and plasma cell antibody production typical of germinal centers[10]. These findings are further supported by the observation that the follicular B lymphocyte centers within the ATLOs show high concentrations of Ki-67 positive proliferating cells, suggesting B lymphocytes are undergoing affinity maturation[7,10] Addtionally, The extensive networks of high endothelial venules, lymph vessels, and blood vessels observed in these ATLOs facilitate the recruitment of immune cells to these developing lymphoid organs[10].

Several chemokines related to the recruitment of lymphocytes likely orchestrate the initiation and further organization of these adventitial lymphoid structures. Expression of CCL21 is known to be important in the development of lymphoid follicles with segregated T and B cell areas as well as extensive networks of lymphatic vessels and high endothelial venules in the thyroid[21,22], and medial smooth muscle cells acting as lymphoid tissue organizers in aged apoE−/− mice produce CCL21 when activated through lymphotoxin β-receptor (LTβR) signalling[10]. Furthermore, CCL21 and CCL19 gradients help mediate the movement of follicular B cells to T cell compartments within lymphoid follicles, and expression of CCR7, their receptor, is upregulated on B cells upon exposure to antigen[10,23]. CXCL13 has likewise been shown to be produced by LTβR-activated medial smooth muscle cells in Apoe−/− mice[10] and is known to be produced by follicular dendritic cells. Together with its receptor CXCR5, which is present on all mature B cells[24], CXCL13 likely plays a crucial role in the recruitment of B cells from the vasculature into the developing adventitial lymphoid aggregate. Interestingly, Muniz and colleagues recently showed that depletion of $CD11c^+$ dendritic cells inhibited LTβR signalling-mediated lymphangiogenesis in thyroid tertiary lymphoid structures, suggesting that lymphatic vascular development may depend on dendritic cell recruitment and activation[25]. It is likely that dendritic cells in developing ectopic adventitial lymphoid structures likewise mediate the development of follicular lymphatic vasculature, which raises the interesting possibility that chemokines responsible for dendritic cell recruitment, such as CCL2, CXCL10, and CXCL9[22] may be important in the progression of ATLO development[25].

T lymphocytes in the Adventitia and in Atherosclerosis

The role of T lymphocytes in the development and progression of atherosclerosis has been an area of active investigation[26,27]. T lymphocyte subsets and their interaction with other inflammatory cells within the adventitia, especially within ATLOs, is an area of great interest[10,28]. Lymphocytes populate the adventitia as previously shown via molecular imaging[29]. Figure 2 depicts an image of T lymphocytes aggregating in the aortic root adjacent to an atherosclerotic plaque in an $Ldlr^{-/-}$ mouse fed a Western diet for 16 weeks. This aggregation of T lymphocytes and adventitial macrophages may represent the beginning formation of an ATLO as it borders the external elastic lamina.

Adventitial T lymphocytes are present early in the development of atherosclerosis, in loose aggregates[6,18]. Much is known about the role of various T lymphocyte subsets, including CD4+ T lymphocytes, CD8+ T lymphocytes, regulatory T lymphocytes, Th-17 cells, and natural killer T cells in atherosclerosis[20,26,27,30,31], with a focus on their role in the developing intima, and this has recently been nicely reviewed[26,27,32]. Adoptive transfer of CD4+ T lymphocytes specifically reactive to oxidized low density lipoprotein (oxLDL) leads to a greater increase development in atherosclerosis than naïve CD4+ T lymphocytes or CD4+ T lymphocytes from mice immunized with keyhole limpet hemocyanin (KLH)[33], suggesting that CD4+ T lymphocytes play a key role in mediating specific antigen-driven responses during atherosclerosis development[33]. Additional studies demonstrating T lymphocytes in the atherosclerotic plaque specific for not only oxLDL but also for heat

shock protein supports the concept that multiple antigens may activate T lymphocytes and other immune cells in the artery wall.[34,35] In a recent study, Hermansson and colleagues demonstrated that T cell hybridomas generated from human ApoB100 transgenic mice responded with production of IL-2 upon in vitro exposure of native LDL and purified ApoB100[36]. These T cells were found to be CD4+ and expressed a lone T cell receptor variable β chain, TRBV31. Interestingly, serological analysis of these mice revealed the presence of IgG antibodies against oxLDL, suggesting that these T cells reactive to native ApoB100 may induce B cells to produce antibodies against oxLDL[36]. Therefore, more work is needed to fully understand the important autoantigens stimulating responses that modulate atherosclerosis development and the specificity of the autoantibodies that they produce. However, clearly CD4+ T lymphocytes can undergo activation and clonal expansion in response to plaque antigens[34] which could lead to local autoantibody production. Yet, the role of ATLOs in this process is poorly understood. Production of cytokines is another mechanism whereby T lymphocytes regulate atherosclerosis formation, and adventitial T lymphocytes could impact intimal plaque development by passage of these cytokines through the channels connecting the adventitia with the media.

IL-17A producing T cells are also present in the adventitia and have been implicated in atherogenesis. Blockade of IL-17A led to a significant reduction in aortic CXCL1 expression, aortic macrophage accumulation and atherosclerosis[31]. Follow on studies to further explore the role of these Th-17 cells in the adventitia are needed. ATLOs have also been shown to harbor a large number of $Fox3P^{+}$ Treg that are predominantly found within the T lymphocyte region[10]. It appears there are significantly more Tregs present in ATLOs surrounding well established plaques than there are in early loose adventitial T lymphocyte aggregates[6,10], yet Tregs have been shown to be atheroprotective[37] through complex interactions with transcription growth factor-β and IL-10[26]. Further studies are needed to characterize the complex interactions of T lymphocyte subsets, cytokines, chemokines, and other inflammatory cells in the adventitia.

B lymphocytes in the Adventitia and in Atherosclerosis

Multiple autopsy studies have reported the presence of B lymphocytes in both the plaque and adventitia surrounding atherosclerotic lesions with a predominance of B lymphocytes found in the adventitia[7,11,38]. Though the contributions of T lymphocytes, macrophages and other proinflammatory cells have been well studied, the role of B lymphocytes in atherogenesis is less well understood. B lymphocyte aggregates have been observed within the nodular centers of adventitial ATLOs and may undergo selection, maturation, and antibody production following antigen presentation by dendritic cells[7,10]. Real-time PCR performed on directional artherectomy samples obtained from coronary arteries of six patients with coronary artery disease demonstrated evidence of antigen-driven clonal expansion and evolution of B lymphocytes in human atherosclerotic plaques[39]. Additionally, in their survey of inflammatory cell infiltration in atherosclerosis, Parums and Mitchinson noted that advanced plaques with evidence of medial attenuation showed significant adventitial plasma cell and B lymphocyte accumulation[19,40]. Plasma cells have been demonstrated in the adventitia around ATLOs[10] and in the coronary arteries in the setting of transplant vasculopathy[41], providing further evidence that B lymphocytes may differentiate into plasma cells within the adventitial environment. These studies establish the presence of activated B lymphocytes in diseased arteries, but fall short of identifying a functional role for B lymphocytes in atherogenesis.

Some of the first data to address a functional role for B lymphocytes in atherosclerosis was published in 2002 by two groups[42,43]. Caligiuri and colleagues[42] demonstrated that splenectomy increased atherosclerosis development in response to 12 weeks of Western

feeding in *Apoe*$^{-/-}$ mice. As splenectomy results in a significant decrease of both B and T lymphocytes, they performed adoptive transfer of purified splenic B lymphocytes into splenectomized *Apoe*$^{-/-}$ mice to determine a role specifically for B lymphocytes. Indeed, adoptive transfer of B lymphocytes into splenectomized *Apoe*$^{-/-}$ mice resulted in a 90% reduction in lesion area compared with controls. Additionally, they found that adoptive transfer of B lymphocytes to non-splenectomized mice also led to a 30% reduction in atherosclerosis, suggesting an atheroprotective role for B lymphocytes. That same year, Major et.al. also demonstrated the atheroprotective properties of B lymphocytes using a different approach. They performed bone marrow reconstitution of *Ldlr*$^{-/-}$ mice with bone marrow from either B lymphocyte-deficient μMT mice or C57Bl/6 mice[43]. The μMT mice lack peripheral B lymphocytes due to a deletion of genomic DNA sequences that encode the transmembrane domain of the B cell receptor μ heavy chain[44]. The μMT bone marrow recipients developed a significant 2-fold increase in atherosclerosis after only 4 weeks of Western feeding compared with C57BL/6 bone marrow recipient mice. This difference remained present even following 12 weeks of Western diet. Taken together, these studies suggested an atheroprotective role for B lymphocytes.

Two more recent studies further expanded our understanding of the role of B lymphocytes in atherosclerosis. Ait-Oufella and Kyaw demonstrated that B lymphocyte depletion with CD20-specific monoclonal antibody resulted in a reduction of atherosclerosis in both *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice[45,46]. Further, consistent with these findings, injection of splenic B2 lymphocytes from C57BL/6 mice into *Apoe*$^{-/-}$ *Rag-2*$^{-/-}$ mice (both B and T lymphocyte deficient) and *Apoe*$^{-/-}$ μMT (B lymphocyte deficient) resulted in aggravation of atherosclerosis[46]. Interestingly, depletion of mature B lymphocytes with CD20-specific monoclonal antibody diminished pathogenic T lymphocyte activiation with reduced IFN-γ secretion and IL-17 production[45]. Taken together, these studies provide evidence for an atherogenic role for B lymphocytes. Notably, CD20 monoclonal antibody treatment depletes B2 lymphocytes more substantially than B1 lymphocytes[45,47] and adoptive transfer of B1 lymphocytes to *Apoe*$^{-/-}$ *Rag-2*$^{-/-}$, unlike B2 cells, did not aggravate atherosclerosis[46]. Moreover, follow-on studies by Kyaw and colleagues recently provided the first direct evidence that B1a lymphocytes are atheroprotective[48]. Consistent with previous reports[49], they demonstrated that splenectomized Apoe$^{-/-}$ mice had a marked depletion of B1a lymphocytes in the peritoneal cavity and an increase in atherosclerosis[42,48]. Moreover, splenectomized Apoe$^{-/-}$ mice had reduced IgM in plasma and in atherosclerotic lesions[48]. Adoptive transfer of B1a but not B2 lymphocytes into splectomized mice restored plasma levels and lesion IgM deposits and reduced the size of atherosclerotic lesions and the prevalence of necrotic cores[48]. Intriguingly, B1a cells lacking the ability to secrete IgM were not able to attenuate the development of atherosclerosis upon adoptive transfer to splenectomized Apoe$^{-/-}$ mice, identifying IgM production as important to B1a lymphocyte-mediated atheroprotection[48]. In support of secreted IgM as important in B lymphocyte-mediated atheroprotection is the recent study by Lewis and colleagues demonstrating that *sIgM*$^{-/-}$ *Ldlr*$^{-/-}$ mice deficient in serum IgM developed larger and more complex atherosclerotic lesions with increased apoptosis in aortic root lesions in response to both low and high-fat diets[50]. Moreover, a wealth of data provided by the Witztum group and others demonstrated that IgM natural antibodies, such as IgM-E06, bind oxidized lipoproteins, block uptake of oxLDL by macrophage scavenger receptors, and attenuate atherosclerosis[51–55]. Taken together, results suggest that the effect of B lymphocytes on atherosclerosis is subset dependent with B2 lymphocytes promoting atherosclerosis and B1 lymphocytes attenuating atherosclerosis. The importance of B lymphocyte subsets and the potential for the therapeutic manipulation of B lymphocyte populations in atherosclerosis has recently been reviewed[56].

Immune cell infiltrates in the adventitia in humans

Though the presence of lymphocytes in the adventitia in humans has been documented since the early 1960's, the natural question of whether fully-developed ATLOs similar to those characterized in mice also develop in humans has not been thoroughly explored. In the setting of advanced atherosclerotic abdominal aortic aneurysms, adventitial lymphoid follicles with an abundance of B lymphocytes and T helper cells that stain positively for the proliferation marker, Ki-67, have been reported[19,57]. These follicles also react with peanut lectin, indicating that they were likely germinal centers of B cell origin. Although these characterizations fall short of demonstrating the intricate high endothelial venules, lymph vessels, and blood vessels, demonstrated by Grabner and colleagues, they do provide compelling evidence that at least some of the adventitial lymphoid organizational characteristics observed in mice may be applicable to humans. Additionally, since atherosclerosis of the coronary arteries is the major clinical problem linked to the mortality associated with atherosclerosis, it is important to consider whether findings of immune regulation from studies in the aorta are also applicable to the coronary arteries. Studies in human coronary arteries have shown adventitial lymphoid infiltrates similar to those present in the human aorta[58]. While the vasa vasorum of noninflammed human coronary arteries do not form a dense capillary plexus[59], like the aorta, with intimal thickening and the progression of coronary artery lesions, the vasa vasorum grow to form a dense network with projections through the media into the intima[59], allowing recruitment of immune cells. Further studies are needed to fully characterize the immune cell environment in the adventitia surrounding coronary arteries in humans.

Intimal/Adventitial Communication

Though much of the research on atherogenesis has focused on the intimal environment, increasing evidence supports the idea that the adventitia may influence the progression of intimal lesions[3–5,60–63]. Several studies suggest that an intact adventitia is necessary for normal intimal endothelial morphology. Surgical removal of the vasa vasorum and subsequent adventitial ischemia results in abnormal intimal endothelium[61,62], and restriction of vasa vasorum blood volume through ligation of the intercostal arteries leads to intimal necrosis[63]. Furthermore, adventitial injury in both the setting of percutaneous coronary angioplasty[3,4] and placement of a circumferential silastic collar[5] leads to neointimal formation. Together, this evidence indicates that communication exists between the intima and adventitia and that the adventitia plays a role in plaque development.

Collagenous Conduits

A significant contribution to our understanding of potential mechanisms whereby cells in the intimal and medial compartments in the vessel wall may regulate and be regulated by the adventitia came when Grabner and colleagues demonstrated the presence of a dense reticular network of collagenous conduits connecting the medial layer of the vessel wall with the underlying adventitia[10]. In studies using intravenous injection of fluorescently labeled dextran particles, they elegantly demonstrated that, like lymph node conduits, these adventitial conduits could transport small (10kD), but not large (500 kD) particles into the medial layer of the vessel wall. These conduits could serve as a possible means for the transport of soluble chemokines or antigens between the activated medial smooth muscle cells or intimal plaque and adventitial cells. Notably, these conduit networks are similar to those known to facilitate lymphocyte organization in the white pulp of the spleen[64] and to those that facilitate the filtering of soluble substances within lymph nodes[65] The observation that ATLO development parallels the severity of the intimal plaque suggests that crosstalk between the intima and adventitia is likely. Lötzer and colleagues described how activation of the LTβR and tumor necrosis factor receptor subfamily, member 1A stimulates medial

smooth muscle cells to transform into cells resembling lymphoid tissue organizers that interact with the adventitial inflammatory infiltrates and may play a role in generating the ATLO organizational structures already discussed[66].

Vasa vasorum

Another way in which the adventitia communicates with the developing intima is through the vasa vasorum. Studies in dogs and humans have shown that in the noninflammed aorta, the vasa vasorum network acts to nourish the outer segments of the vessel wall and the outer two thirds of the media, while the intima and inner third of the media is fed via passive oxygen diffusion through the luminal endothelium[67,68] Caution must be exercised when extrapolating data on immune cell recruitment to the vessel wall in mice to humans as the vessel wall thickness in mice is small enough that the adventitial vasculature may adequately perfuse a large portion of the normal vessel wall. However, with the progression of atherosclerosis in humans, oxygen diffusion is impaired and the vasa vasorum grows to become the primary source of nutrients for the entire vessel wall[69,70]. These intimal projections of the vasa vasorum have long been known to be important in immune cell recruitment to intimal plaques in humans[71,72]. Thus, the vasa vasorum may have similar functions in the migration of lymphocytes to the adventitia and subsequent modulation of the immune response in atherosclerotic plaques in humans and mice.

In addition to enabling cellular migration to the intima[20], the vasa vasorum likely plays a key role in the migration of lymphocytes to the adventitia and subsequent modulation of the immune response in atherosclerotic plaques. T lymphocytes have been observed entering adventitial immune tissues via the vasa vasorum[18,29], and this may be facilitated by increased expression of integrins and selectins critical for inflammatory cell recruitment in microvessels[73]. Furthermore, microvessels in the proximity of adventitial inflammatory cell clusters have been shown to be reactive to HECA-452 and peripheral lymph node addressin (PNAd), both markers of specialized high endothelial venules (HEVs) by which lymphocytes enter lymphoid tissue, These findings provide evidence that the structures needed for lymphocyte trafficking into functional lymphoid aggregates are present in the adventia[7,10]. HEVs were also shown to enable the migration of lymphocytes from the vasculature into lymphoid tissue in cases of chronic inflammation[74], suggesting these microvessels may be necessary for the development of ATLOs and subsequent immune modulation of atherosclerosis development. It is possible that the conduits described by Grabner and colleagues allow for the transport of antigens, cytokines and chemokines between the intima and adventitia that subsequently promote neovascularization of the vasa vasorum and the coordination of cells recruited through the vasa vasorum into the tertiary lymphoid structures described earlier.

Perivascular adipose tissue

The complex interplay of inflammatory and anti-inflammatory cytokines and adipokines produced within perivascular adipose tissue (PVAT) and their impact on the atherosclerotic plaque has previously been reviewed[75]. Though many of the cardiovascular risk factors associated with atherosclerosis lead to an increase in the development of PVAT, a recent noninvasive computed tomographic study of patients demonstrated that doubling of a patients pericoronary fat volume resulted in a 2.5-fold increase in the presence of atherosclerotic plaque in the underlying coronary segments, and this remained significant following adjustment for traditional cardiovascular risk factors and overall pericardial fat volume[76]. PVAT surrounding these atherosclerotic plaques intriguingly demonstrates a marked increase in inflammatory cell density[77]. Additionally, it is important to recognize that PVAT surrounds the adventitia with no limitation on cellular trafficking between the two tissues. As PVAT increases in size with increasing development of atherosclerosis[76],

the increased inflammatory cell burden may easily traffic to the adventitia and play a role in developing inflammatory responses. Interestingly, PVAT-derived oxidative stress has been shown to play a major role in endothelial dysfunction of the vasa vasorum[78,79] and may also provide a stimulus for formation of microvessels. Additionally, vasoactive factors produced by PVAT[79,80] and other adipokines and inflammatory chemokines may not only modulate microvessel function but also lead to increased recruitment of lymphocytes[81] Interestingly, in addition to a role in intimal plaque progression, PVAT lymphocytes have also been implicated in the regulation of blood pressure. Angiotensin II infusion increased the number of T cells in the PVAT, T cell production of TNFα, and the amount of both intercellular adhesion molecule-1 and RANTES in the aortic wall, while TNFα agonists hindered the hypertensive response to angiotensin II[82]. Thus, the complex interplay of PVAT, lymphocytes, and the adventitial vasa vasorum, may impact not only atherogenesis, but other common vascular disorders.

Disruption of the medial barrier

Immunohistochemical studies of atherosclerotic lesions demonstrating accumulations of lymphocytes and macrophages in the intima and the adventitia but not the media[83–86] suggest that the media normally acts as a barrier to the trafficking of phagocytes and lymphocytes between intima and adventitia. It has been suggested that the elastic laminae bordering and within the medial layer may provide a physical barrier to lymphocyte trafficking through the media. In addition, interesting studies by Cuffy et.al. and Dal Canto et.al. suggest that the media of the vessel wall may be “immunoprivileged”, inhibiting immune cells from trafficking through it[87,88]. Initial insights into this phenomenon came with the study by Dal Canto and colleagues. They developed an animal model of chronic vasculitis by infecting IFN-$\gamma R^{-/-}$ mice with γ-herpesvirus 68, noting that the chronicity of the infection was due to the inability of T cells and macrophages to infiltrate the infected media and clear the γ-herpesvirus 68[88]. Follow on studies demonstrated that medial VSMC produced indoleamine 2,3-dioxygenase (IDO), a factor that mediates immune privilege of the fetus cohabitating within the mother[87]. Inhibition of IDO by 1-methyl-tryptophan increased medial infiltration by allogeneic T cells suggesting that cytokines produced by VSMC inhibit immune cells from trafficking into the media[87]. Whether as a physical barrier or due to active anti-inflammatory mechanisms such as production of IDO, the healthy intact media appears to serve as barrier to immune cell trafficking between the intimal and advential compartments. The collagenous conduits and the vasa vasorum provide a method to bypass this barrier and allow communication between these compartments by allowing cytokines, chemokines, and cells a path through the media. In addition to these pathways, disruption of the media itself may allow for greater communication. As early as 1981, Parums and Mitchinson recognized that advanced plaques surrounded by an intact media typically elicit little adventitial inflammation, while medial thinning around advanced plaques was associated with more prevalent adventitial inflammatory infiltrates[40]. In this setting, dendritic cells and macrophages may migrate from the plaque to the adventitial inflammatory foci, where they subsequently could serve as antigen presenting cells[89,90]. It has previously been suggested that medial attenuation allows normally sequestered antigens within the intima to be recognized by lymphocytes in the adventitia[19]. Activation of adventitial immune cells due to compromise of the medial barrier could lead to further release of cytokines and chemokines that could travel to the intima via conduits and enhance inflammatory cell recruitment and intimal lesion formation. The breakdown of the media itself in disease states such as advanced atherosclerosis (Figure 1), arterial injury[4,91] and arterial aneurysm provide a context in which the physical barrier of the media could be so compromised that it allows direct cellular trafficking. Studies in porcine models of vascular injury demonstrate that medial disruption results in migration of adventital cells into the intima[4,91].

Abdominal aortic aneurysms (AAA) are perhaps the most well-known settings of medial breakdown. Though our understanding of the mechanisms leading to AAA continue to evolve, inflammation and matrix degradation are clear hallmarks of its development, and inflammation appears primarily in the adventitia and outer media of the vessel wall[60]. AAA has long been associated with atherosclerosis, but it is important to recognize that the severity of atherosclerosis does not correlate with AAA development[92]. Notably, not all patients with severe atherosclerosis develop AAA, and not all patients with AAA are afflicted with severe atherosclerosis, suggesting that the two can develop in a parallel response to common risk factors instead of in a causative manner. It is well known that macrophages in the setting of AAA produce several factors that contribute to the degradation of the extracellular matrix, including cathepsins[93], and multiple matrix metalloproteinases[94–96]. A similar process occurs in atherogenesis, where macrophages produce matrix degrading enzymes, leading to medial barrier disruption and subsequent increased communication between the intima and adventitia[97].

Immune cells in the Adventitia of Non-diseased Arteries

Adventitial immune infiltrates that develop in association with advanced intimal atherosclerotic plaques have been well characterized[10,18]. However, the existence and function of resident immune cells in the aorta is less well understood. Galkina and colleagues provided important insight into this question through studies utilizing flow cytometry which enabled quantitiative analysis of the number and percentage of leukocytes in the vessel wall. Through their carefully controlled studies, they demonstrated the presence of macrophages, dendritic cells, T lymphocytes and B lymphocytes in the normal, non-inflamed aortas in C57BL/6 mice, indicating that there is a resident population of leukocytes in the vessel wall regulated by constitutive trafficking[18]. Immunohistochemical data from the same study provided evidence that many of these leukocytes, including the lymphocytes, were present in the adventitia. Using en face immunoconfocal microscopy, Jongstra-Bilen and colleagues demonstrated that the adventitia of normocholesterolemic 3–6 month old C57BL/6 mice contain accumulations of T cells and macrophages, providing further evidence for a constituitive immune cell presence in the non-inflamed adventitia[98]. Mice on the atherogenic $Apoe^{-/-}$ background have greater numbers of baseline aortic immune cells than C57BL/6 mice even prior to Western diet feeding. These numbers are greater in the absence of visible intimal lesions, suggesting adventitial accumulation. Of note, while total leukocyte numbers in the aorta of $Apoe^{-/-}$ mice are greater than in C57BL/6 mice, the percentage of specific leukocyte cell types differs. $Apoe^{-/-}$ have an increase in the percentage of macrophages and dendritic cells compared to C57BL/6 mice, but no change in the percentage of T cells and reduced B cell percentages[18], suggesting a link between the type of adventitial immune cells and propensity of the mice to develop atherosclerosis. It is intriguing to hypothesize that the immune milieu of the normal vessel may be present to respond to early atherogenic signals and perform innate anti-inflammatory functions which become altered in the setting of chronic hyperlipemia ($Apoe^{-/-}$ mice) and overwhelmed in the setting of marked hyperlipidemia (addition of Western diet) or other vascular insults. Of note, the greatest percentage of B lymphocytes is seen in the aorta of C57BL/6 mice. Moreover, studies in $Apoe^{-/-}$ mice with deletion of the helix-loop-helix factor Inhibitor of Differentiation 3 (Id3) which results in significantly reduced number of B cells in the aorta reveals significantly more atherosclerosis compared to $Apoe^{-/-}$ controls in response to Western diet feeding[99]. Atherosclerosis is present in $Id3^{-/-}Apoe^{-/-}$ mice as early as 4 weeks after Western diet feeding, raising the intriquing hypothesis that B lymphocytes resident in aortic adventitia may limit early and exaggerated atherosclerosis. Notably, a functionally significant polymorphism in the human Id3 gene is associated with increased intima medial thickness in humans[100]. Indeed, analysis of human vessels also supports the existence of resident adventitial leukocytes. Immunohistochemical studies in aortas of children prior to

the development of atherosclerosis have reported leukocytes surrounding the vasa vasorum in the adventitia[20]. However, more studies of the adventitia of normal human arteries is needed to fully characterize the immune cells present.

Imaging Lymphocytes and the Adventitia in Atherosclerosis

Molecular and cellular imaging can characterize lymphocyte biology in vivo[101–104105], and several of these, and other approaches can and have been applied to elucidating leukocyte biology in the adventitia. For example, intravital microscopy has enabled clear visualization of the process of leukocyte rolling and adhesion on the vascular endothelium[106], serving as a potentially powerful technique to study lymphocyte interactions within small vessels. Intravital microscopy was used to demonstrate the importance of P-selectin in leukocyte adhesion to the endothelium, as P-selectin-deficient mice have significantly reduced leukocyte recruitment and attenuated inflammatory disease[101]. Two-photon intravital microscopy can even visualize the interactions of living cells deep within tissues without significant concern for phototoxicity or photobleaching[107,108]. Detection of adhesion molecule expression using synthetic optical probes can also be used to identify key molecules in the adventitia regulating leukocyte recruitment and adventitial growth. Fluorophore binding of an αvβ3 integrin targeted peptide identified marked expression of this adhesion molecule in the adventitia of hyperlipidemic rabbits that correlated with adventitial thickness[109]. In vivo imaging of this and other synthetic optical probes with advanced imaging modalities could provide key insights into factors regulating the expression of these molecules in the adventitia throughout the course of disease. Additionally, lymphocytes can be engineered to study their role in the immune system through production of bioluminescence activity or detection via positron emission tomography following specific gene activation[102–104]. Galkina et. al. utilized a mouse model where green fluorescent protein (GFP) was knocked in to the CXCR6 locus to determine that T lymphocyte recruitment to the vessel wall is mediated by CXCR6[110]. These T lymphocytes are predominantly found in the adventitial layer[6,18]. Multiphoton microscopy of the carotid artery of Apoe$^{-/-}$ mice indeed demonstrated that adoptively transferred labeled lymphocytes were mainly found in the adventitial layer[29]. Noninvasive optical imaging of luminescent proteins could be applied to help determine the site and timing of lymphocyte recruitment and activation during the course of atherogenesis. Similarly, multimodality contrast agents can be targeted to molecules on lymphocytes and determine not only functional characteristics but also identify and track certain lymphocyte subsets[111105,112].

Simple imaging techniques have recently led to the novel finding that B lymphocytes home to regions prone to and with existing atherosclerosis. Initial imaging, validated by flow cytometry data, confirmed that B lymphocytes were present in non-diseased aorta[99]. Quantitation of near-infrared fluorescence-mediated tomographic imaging of Cy5.5 conjugated to CD19 or B220-specific antibodies demonstrated B lymphocytes in the aorta of *Apoe*$^{-/-}$ mice fed a chow diet. The B lymphocyte-deficient μMT *Apoe*$^{-/-}$ mouse was used as a control for nonspecific signal. There was significantly greater fluorescence present in the aorta of the *Apoe*$^{-/-}$ mouse when compared with the aorta from the μMT *Apoe*$^{-/-}$ mouse. Interestingly, this technique also provided evidence that B lymphocytes were predominantly detected in regions of the aorta prone to the development of atherosclerosis (arch and abdominal aorta). Phosphor imaging of B lymphocytes radiolabeled with indium-111 oxyquinoline and adoptively transferred into μMT *Apoe*$^{-/-}$ mice demonstrated constituitive homing of B lymphocytes to these very same regions. Moreover, transfer of B lymphocytes radiolabeled with indium-111 oxyquinoline to μMT *Apoe*$^{-/-}$ mice with existing early atherosclerosis revealed that B lymphocytes trafficked predominantly to regions of lipid deposition[99]. Advantages of adapting this technique to perform imaging of

radiolabeled immune cells are the ability to isolate and label specific cell types, the high sensitivity afforded by the modality, and the lack of impact on cellular function, While labeling specific cells via surface molecules may alter cellular function, non-specific radiolabeling with indium-111 oxine is unlikely to significantly impact cell surface receptor expression, cellular trafficking or function[113,114]. The technique could have been further improved through incorporation of radioautographic microscopy, which would have helped determine the location of the radiolabeled cell within the layers of the artery. To complement this approach, labeling lymphocytes with CFSE or other analogues have been used. Histology of aortic sections after transfer of labeled B cells demonstrate that they trafficked to the adventitia[18,99].

Finally, the characterization of the adventitial vasa vasorum network has also provided significant insight into potential clinical imaging techniques. Contrast-enhanced ultrasound has demonstrated that increased contrast in the adventitia strongly correlates with adventitial vasa vasorum and the progression of atherosclerosis[115–118]. Contrast-enhanced magnetic resonance imaging provides another means of quantifying the presence of vasa vasorum in atherosclerotic lesions[119]. Furthermore, multimodality imaging with MRI and positron emission tomographic imaging enabled highly sensitive detection of neovessels within the plaque and the necessary spatial resolution to distinguish intimal neovessels from the adventitial vasa vasorum[120]. The ability to target adhesion molecules expressed on the endothelium may also enable early detection of neovascularization and characterization of atherosclerosis[121]. These imaging agents can also be readily modified to deliver therapeutic agents that can disrupt the formation of vasa vasorum and attenuate the progression of atherosclerosis[122]. Thus, the same imaging agents used to perform targeted imaging can also serve as a vehicle to provide targeted drug delivery. Molecular and cellular imaging can be a powerful tool to characterize and study atherosclerotic plaque in animals[13], Many of these techniques also hold the potential to provide structural characterization of molecules and cell types present in all layers of the artery wall in patients throughout the course of atherosclerosic lesion development. The current knowledge on molecular imaging of atherosclerosis in clinical practice has been thoroughly reviewed[123,124]. These advanced imaging techniques may help in the early identification of patients with atherosclerosis who are at risk for rapid lesion progression or plaque rupture and subsequent acute atherothrombotic events.

Conclusion

Evidence continues to mount highlighting the importance of lymphocytes and the adventitia in the coordination of the immune response in atherosclerosis. As illustrated in Figure 3, inflammatory chemokines, cytokines, and growth factors from the atherosclerotic plaque in the intima result in neovascularization arising from the adventitial vasa vasorum, accumulation of adventitial T and B lymphocytes which organize along with other inflammatory cells to form early immune tissues and eventually give rise to form ATLOs. ATLOs may serve as sites for immune responses to plaque antigens such as HSP60 or modified lipoproteins. Resident immune cell accumulation in the adventitia and PVAT may play a role in innate protection from atherogenic antigens or cytokines. Finally, PVAT may modulate inflammation through migration of immune cells in the PVAT into the adjacent adventitia and transfer of adipokines, chemokines, and cytokines from local adipocytes across the media via conduits and microvessels. While further studies are necessary to fully characterize the importance of the adventitia in regulating the immune response to atherosclerosis, it is clear that we can no longer view atherosclerosis as a disease of the intima but recognize it as an inflammatory disease of the entire arterial wall.

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Non-standard Abbreviations and Acronyms

AAA	Abdominal Aortic Aneurysm
APC	Antigen Presenting Cell
ATLO	Aortic Tertiary Lymphoid Organ
Ig	Immunoglobulin
IFN-γ	Interferon gamma
IL	Interleukin
MIP-1α	Macrophage Inflammatory Protein-1 alpha
MMP	Matrix Metalloproteinase
Th	T Helper

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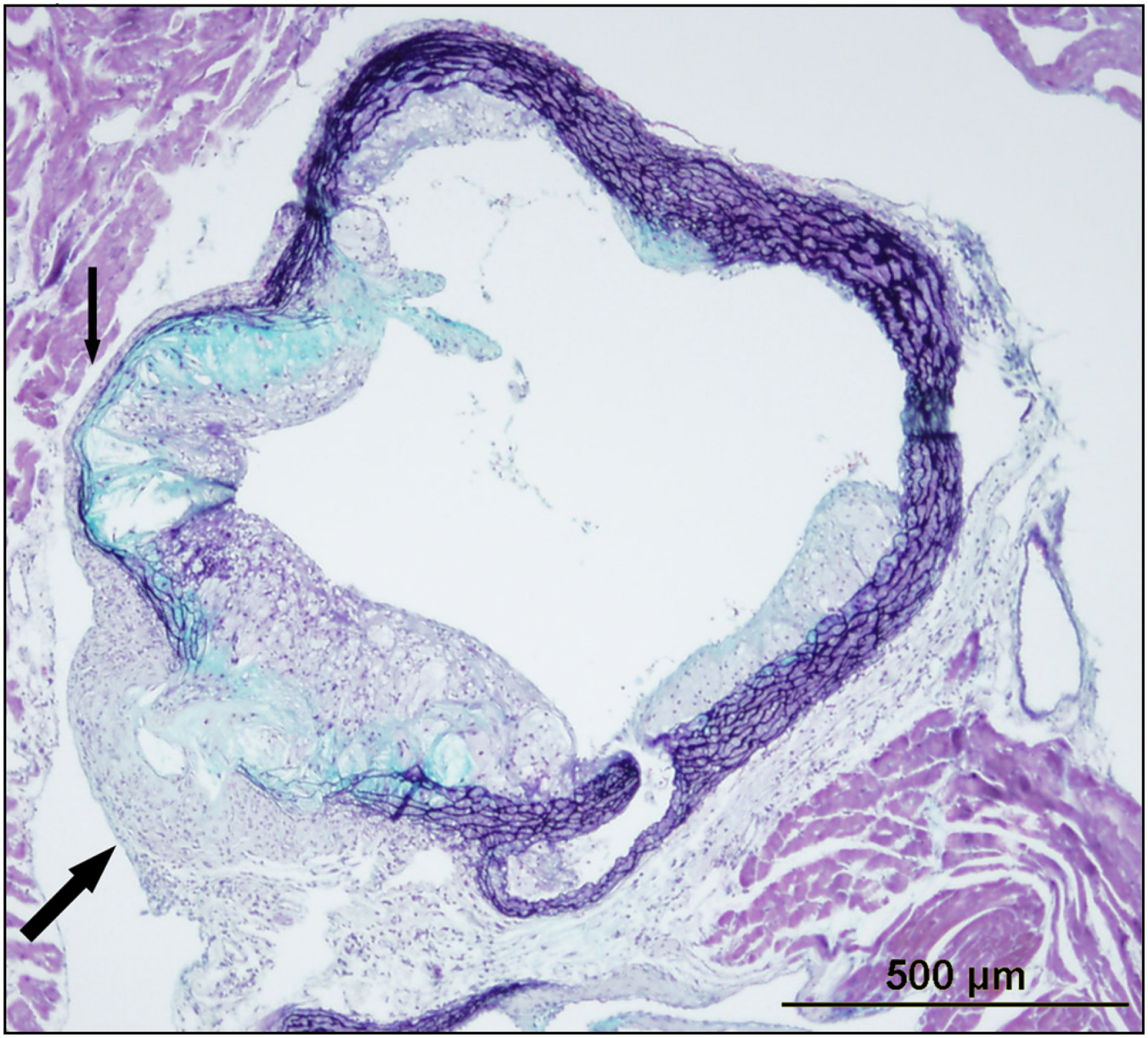

Figure 1. Movat's stain of a cross section of a 24 week old *Ldlr*$^{-/-}$ mouse fed a Western diet for 16 weeks

As indicated by the small arrow, there is thinning of the media and breakdown of the internal and external elastic lamina in the setting of advanced atherosclerotic plaque. The large arrow points to an advanced atherosclerotic lesion that has breached the internal elastic lamina, media, and external elastic lamina with evidence of necrotic core and cholesterol crystals within the adventitia. This breach enables emigration of intimal macrophages, dendritic cells, and lymphocytes to the adventitia and compromises the barrier status of the media. There is compensatory thickening of the adventitia in this region which likely serves to contain the breached media.

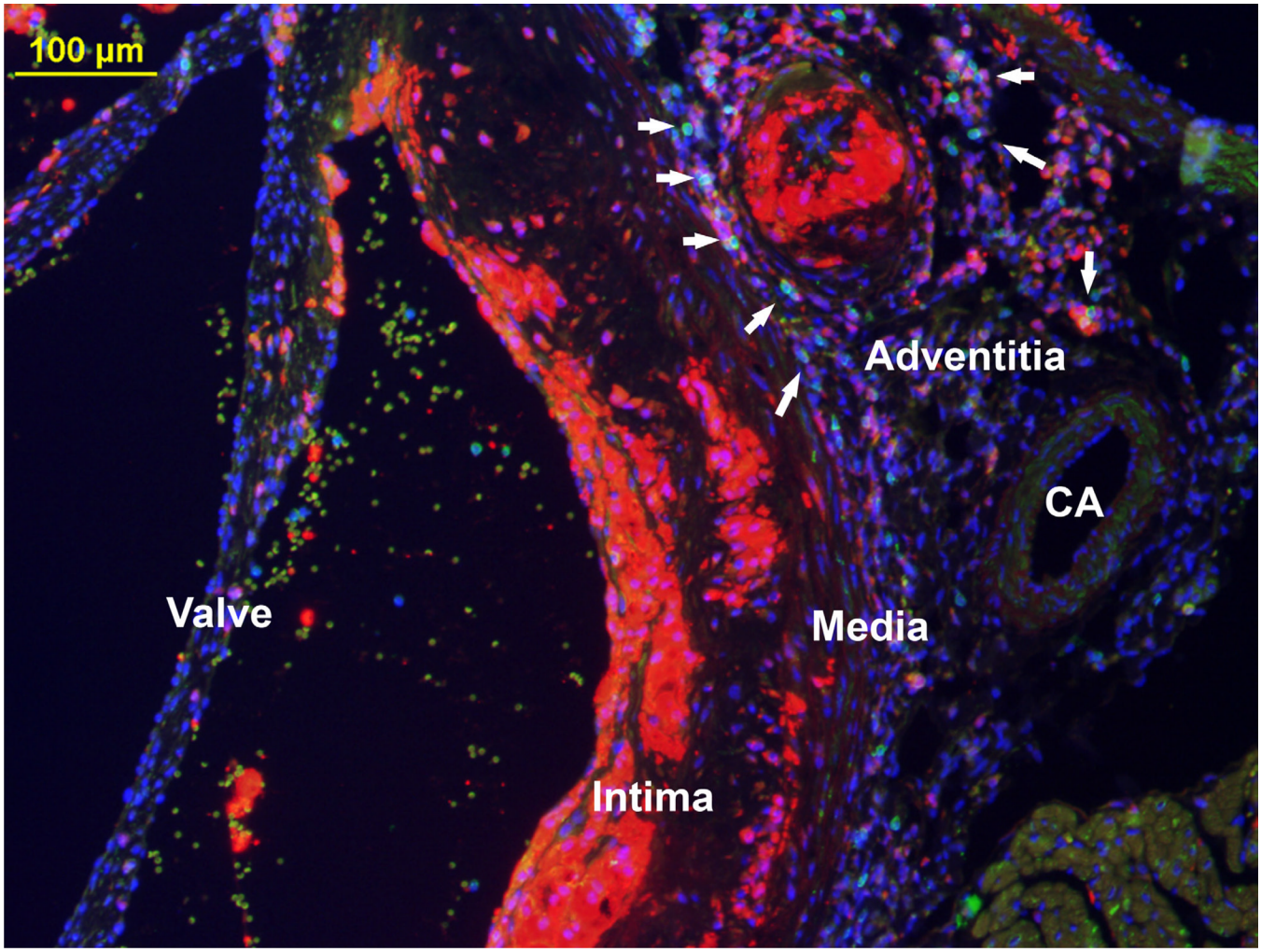

Figure 2. Fluorescent microscopy (10X) of a cross-section from the aortic root of a 24 week old *Ldlr*$^{-/-}$ mouse fed Western diet for 16 weeks

Macrophages appear red (anti-Mac-2), T lymphocytes appear green (anti-CD3), and nuclei appear blue (DAPI). The overlay RGB image clearly demonstrates the intima, media, and adventitia with the presence of an atherosclerotic plaque containing macrophages and T lymphocytes. Adjacent to the coronary artery (CA) is an area with T lymphocytes as well as an area of adventitial macrophages.

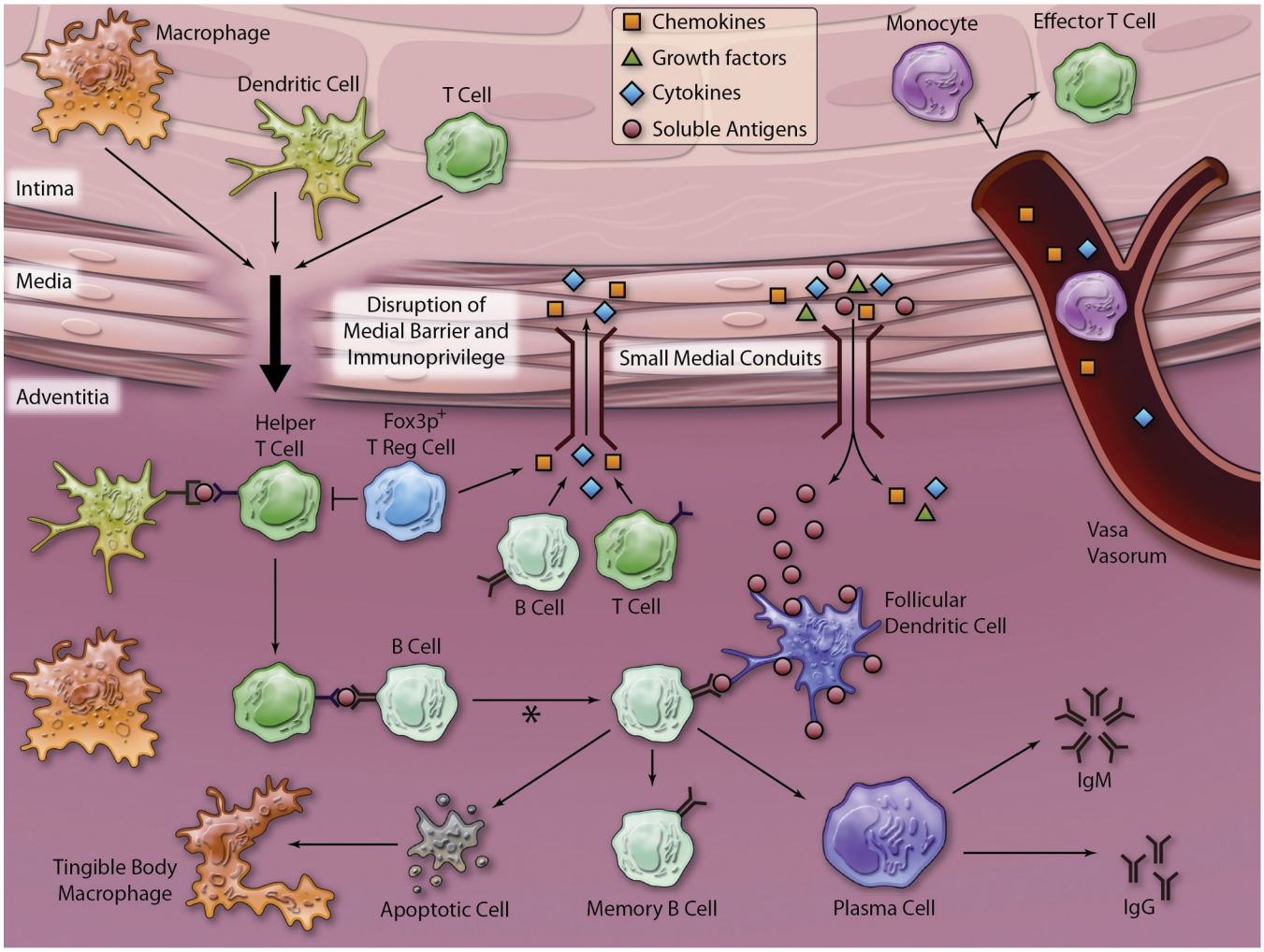

Figure 3. Proposed model of immune system activity in the adventitia

Small medial conduits, as demonstrated by Gräbner and colleagues, may enable soluble antigens, cytokines, growth factors, and chemokines to traffic between the intima and the adventitia. Chemokines and cytokines passing through these conduits may aid in the recruitment of leukocytes to the growing atherosclerotic plaque via the vasa vasorum. Additionally, growth factors secreted by cells within the growing plaque may stimulate neovascularization from the adventitial vasa vasorum. As shown in Figure 1, the media underlying advanced atherosclerotic plaques may be breached, compromising the barrier status of the media. In this model, cells can then cross the media and present antigen to helper T lymphocytes. The helper T lymphocytes (negatively regulated by $Fox3p^+$ T reg lymphocytes) then stimulate B lymphocytes to undergo clonal expansion, isotype switching, and affinity maturation (asterisk). B lymphocytes with low affinity for antigen presented on follicular dendritic cells may undergo apoptosis and be removed by tingible body macrophages via efferocytosis. The B lymphocytes selected through high affinity interaction with antigen presented on follicular dendritic cells may undergo differentiation into memory B lymphocytes and plasma cells that may produce IgM or IgG autoantibodies locally or traffick to lymph nodes.