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Once Upon a Time: The Adaptive Immune Response in Atherosclerosis—a Fairy Tale No More

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Extensive research has been carried out to decipher the function of the adaptive immune response in atherosclerosis, with the expectation that it will pave the road for the design of immunomodulatory therapies that will prevent or reverse the progression of the disease. All this work has led to the concept that some T- and B-cell subsets are proatherogenic, whereas others are atheroprotective. In addition to the immune response occurring in the spleen and lymph nodes, it has been shown that lymphoid neogenesis takes place in the adventitia of atherosclerotic vessels, leading to the formation of tertiary lymphoid organs where an adaptive immune response can be mounted. Whereas the mechanisms orchestrating the formation of these organs are becoming better understood, their impact on atherosclerosis progression remains unclear. Several potential therapeutic strategies against atherosclerosis, such as protective vaccination against atherosclerosis antigens or inhibiting the activation of proatherogenic B cells, have been proposed based on our improving knowledge of the role of the immune system in atherosclerosis. These strategies have shown success in preclinical studies, giving hope that they will lead to clinical applications.

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INTRODUCTION

Atherosclerosis is a chronic and progressive disease that is characterized by the accumulation of lipids, cells and fibrous elements forming atherosclerotic plaques in arteries of medium and large caliber. It can lead to complications such as stroke and myocardial infarction, which are principal causes of death worldwide. Atherosclerosis starts with the accumulation of low-density lipoproteins (LDLs) in the intima (innermost layer) of the artery, where they undergo oxidation leading to the formation of oxidized LDLs (oxLDLs). Macrophages and vascular smooth muscle cells (VSMCs) can take up oxLDLs and transform into lipid-loaded foam cells to form fatty streaks. Fatty streaks then progress into mature atherosclerotic plaques due to further lipid accumulation and leukocyte infiltration forming the core region of the plaque and the formation by VSMCs of a collagen-rich fibrous cap around this core (1,2).

In the last decade, we have achieved decisive progress in our understanding of how the immune system regulates the development of atherosclerotic lesions. The quest to understand the function of the adaptive immune response in atherosclerosis has been pursued by many researchers after the observation that T cells infiltrate human atherosclerotic plaques (3). Some studies suggest that the adaptive immune response is proatherogenic (4–6), and, therefore, would be a logical therapeutic target in atherosclerosis. However, the design of such therapeutic strategies has been slowed down by the fact that the immune response also can be atheroprotective, depending on the players examined. For instance, we published in this journal more than a decade ago that IL-10, an antiinflammatory cytokine, played a protective role in atherosclerosis, as the absence of IL-10 led to an increased atherosclerotic lesion size, thrombosis and a plasma level of LDLs in mice that are prone to develop spontaneous atherosclerotic lesions due to a deficiency in the apolipoprotein E gene ($ApoE^{-/-}$ mice) (7). As a result, a lot of effort has been expended over the past years in deciphering the function of the different players of the immune system in the physiopathology of atherosclerosis.

For a long time, it was assumed that the adaptive immune response, whether protective or proatherogenic, was generated in secondary lymphoid organs (SLOs). This idea was challenged recently by our group's and others' obser-

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vation that organized lymphoid aggregates accumulate in the adventitia (outermost layer) of the artery close to the lesions (8–10). These lymphoid aggregates resemble SLOs, as they present a highly organized structure with T- and B-cell areas, high endothelial venules (HEVs), fibroblastic reticular cell (FRC)-like cells and germinal centers, and were therefore called tertiary lymphoid organs (TLOs). These observations raised a new range of questions: What is the impact of the immune response that is mounted in TLOs versus SLOs on the progression of atherosclerosis? How do these structures form? Can we modify the generation or quality of this local immune response to slow down atherosclerosis?

In this retrospective, we review growing knowledge about the role of T and B cells in atherosclerosis and recent advances on the development and function of TLOs. Then, based on our improving understanding of the generation and role of the immune response in atherosclerosis, we discuss the therapeutic approaches that have been designed.

THE ADAPTIVE IMMUNE RESPONSE IN ATHEROSCLEROSIS: GOOD FAIRIES AND EVIL WITCHES

Conflicting results on the role of the adaptive immune response in atherosclerosis progression come from studies in mouse models of atherosclerosis, such as ApoE$^{-/-}$ mice or mice deficient for the receptor for LDL (LDL-R$^{-/-}$). On the one hand, crossing these mice to Rag-deficient or SCID mice that lack mature T and B cells reduced the size of atherosclerotic lesions, suggesting that the adaptive immune response is proatherogenic (4,5). On the other hand, splenectomy dramatically aggravated atherosclerosis in ApoE$^{-/-}$ mice, suggesting a protective role for B cells (11). These seemingly contradictory observations can be explained by the fact that some subsets of B cells and T cells are deleterious in atherosclerosis, whereas others are protective.

Whereas both CD4$^+$ and CD8$^+$ T cells are present in human lesions (3), most studies have focused on the role of the different CD4$^+$ T-cell subsets in atherosclerosis. Depending on the cytokine microenvironment, naïve CD4$^+$ T cells can differentiate into specialized effector subsets (Th1, Th2, Th17, etc.), producing different sets of cytokines and being involved in helping other immune cell types, or into regulatory T cells (Tregs). Transfer experiments of T cells from atherogenic mice into immunodeficient SCID ApoE$^{-/-}$ mice accelerated atherosclerosis and increased the serum level of the Th1 cytokine IFN-γ (5), suggesting a proatherogenic role of Th1 cells. Further experiments confirmed that Th1 cells, as well as Th17 cells, were deleterious (12–14). We also could demonstrate that a population of unconventional T cells, the NKT cells, plays a proatherogenic role, a finding pointing at glycolipids as putative plaque antigens (15). By contrast, our work on IL-10 in atherosclerosis, a cytokine produced by Th2 and Treg cells, suggested that these subsets might be beneficial (7). Along this line, LDL-R$^{-/-}$ mice deficient for IL-5, another Th2 cytokine, display enhanced atherosclerosis (16) and depletion of CD4$^+$ Tregs-aggravated atherosclerosis (17,18). Skewing the T-cell response to atherosclerotic-associated antigens toward a Th2 profile is therefore a potential therapeutic approach, and was shown to increase serum titers of atheroprotective antibodies (12).

Manipulating the immune response to broadly increase the humoral response might, however, prove risky. Indeed, the humoral response is not always atheroprotective (19,20). In fact, T-independent innate-like B1 cells producing natural immunoglobulin M (IgM) antibodies (against oxLDL for instance) are protective in atherosclerosis (11,16,21,22), whereas classical T-dependent follicular B2 cells are proatherogenic (19,20). Accordingly, in humans, circulating levels of anti-oxLDLs IgM antibodies have been shown to associate with reduced atherosclerosis, whereas levels of anti-oxLDLs IgG antibodies are either positively correlated or not related to atherosclerosis and cardiovascular risk (23,24). Vaccination protocols to boost levels of anti-oxLDL IgM antibodies, therefore, look potentially promising as a therapeutic approach, and mouse studies have corroborated that such approaches protect against atherosclerosis (16,21,25).

Other potential therapeutic targets emerging from these studies are the T-dependent B2 cells. One of the main features of these cells is to differentiate into plasma cells producing high-affinity antibodies following heavy-chain class switching and affinity maturation. If these antibodies react against autoantigens, as is the case in atherosclerosis (26), a potential deleterious autoimmune response ensues. Heavy-chain class switching and affinity maturation processes occur in germinal centers (GCs) and require the help of a specific CD4$^+$ T-cell subset called the follicular helper T (Tfh) cell subset (27). A tight regulation of Tfh cells, notably by Qa-1-restricted CD8$^+$ regulatory T cells, is critical to maintain self-tolerance (28). Given the proatherogenic role of B2 cells, we investigated whether Tfh cells played a role in atherosclerosis progression (29). We observed that Tfh cells and GC B cells expanded during atherogenesis in ApoE$^{-/-}$ mice. Furthermore, in ApoE$^{-/-}$ mice where a point mutation in the Qa-1 molecule (ApoE$^{-/-}$ D227K mice) prevents the interaction between CD8$^+$ Tregs and Tfh cells, Tfh cell and GC B-cell numbers further increased, and atherosclerotic lesions were larger. These data showed that the Tfh-GC axis is proatherogenic and suggested that inhibiting Tfh cells might decrease atherosclerosis. Indeed, treatment of ApoE$^{-/-}$ D227K mice with an anti–inducible T-cell costimulator ligand (anti-ICOSL) antibody, known to impair Tfh cell development (30), normalized lesion size to the level seen in ApoE$^{-/-}$ mice (29).

As a summary, most of the experimental data points to a protective role of B1 cells and natural IgM antibodies and a proatherogenic role of B2 cells and IgG antibodies. However, presuming the function of antibodies based solely on their isotype is probably an oversimplification, and the specificity of the antibody

might also affect its function. For instance, there is no direct evidence that anti-oxLDL IgG antibodies are pathogenic, and they might actually be protective by neutralizing soluble oxLDLs and preventing their uptake by macrophages or by binding to inhibitory receptors (31). Accordingly, vaccination protocols aiming at inducing antibodies against oxLDLs often increase both anti-oxLDL IgM and IgG titers, yet the overall effect is atheroprotective (16,25). Antibodies directed against cellular antigens, such as heat shock protein 60 (hsp60), might, on the other hand, be proatherogenic by inducing the lysis of hsp60-expressing cells such as stressed endothelial cells (32). It is therefore possible that the progression of atherosclerosis is linked to a drift in the humoral response from protective detoxifying/neutralizing antibodies (produced by B1 cells) against oxLDLs, to antibodies (produced by B2 cells) against antigens whose recognition triggers proatherogenic effects.

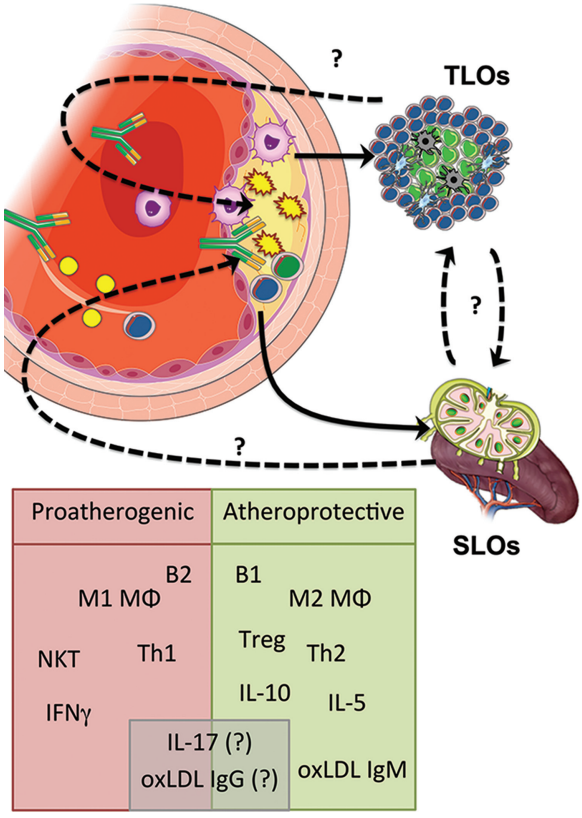

Figure 1. The adaptive immune response in atherosclerosis. Both proatherogenic and atheroprotective players have been identified in atherosclerosis. The immune response can be generated in secondary lymphoid organs (SLOs) such as lymph nodes and spleen, or in tertiary lymphoid organs (TLOs) in the adventitia associated to the lesions. The relative contribution of SLOs and TLOs to atherosclerosis progression, how immune effectors enter the plaque (luminal versus intraplaque neovessels), the relationship between TLO formation and intraplaque/adventitial angiogenesis, as well as the extent of exchange between SLOs and TLOs (are TLOs functionally connected to lymphatics?), are unresolved issues.

WHERE IS THIS ADAPTIVE IMMUNE RESPONSE GENERATED: FARAWAY LYMPH NODES OR NEARBY ADVENTITIA?

In most animal studies of atherosclerosis, the adaptive immune response was looked at in SLOs and the immune effectors were searched for within the plaque. However, the observation of TLOs in the human adventitia of atherosclerotic aorta (10,33), as well as in the adventitia of atherosclerotic mice (8,9), suggests that an adaptive immune response could be mounted within the arterial wall in the adventitia (Figure 1). Indeed, GC B cells, plasma cells and Tfh cells can be found in adventitial TLOs of atherosclerotic ApoE$^{-/-}$ mice, arguing that a humoral immune response might be generated locally (8,29). We recently confirmed that this also is the case in human vascular disease, as the adventitia from atherosclerotic abdominal aortic aneurysms displays TLOs containing Tfh, GC B cells and plasma cells and releases antibodies (29). The existence of this lymphoid neogenesis in the aorta might impair the efficiency of therapeutic strategies targeting the immune system, such as B2 cell depletion, as it occurs in other chronic inflammatory settings such as chronic graft rejection. For instance, rituximab (anti-CD20 antibody) treatment, which depletes circulating B cells, failed to eliminate the intragraft B cells in the context of renal allograft (where TLOs develop in the interstitium of the graft) and local alloantibody production persisted (34). This might be due to the enrichment in B-cell survival factors within the graft inflammatory microenvironment. In atherosclerosis, it might therefore be necessary to design therapeutic approaches that impact the local immune response in the adventitia. To do so, we need to understand how TLOs form.

In contrast to SLOs, which are formed during embryogenesis at the crossroads of lymphatic vessels, TLOs apparently can arise in any peripheral tissue in case of chronic nonresolving inflammation, when the antigen persists, such as chronic infection, autoimmune diseases or transplantation (35). However, several studies have highlighted similarities in the mechanisms driving their formation. For SLO formation, it is crucial that lymphoid tissue inducer (LTi) cells interact with stromal lymphoid tissue organizer (LTo) cells. In mice, LTi cells are CD3^{-}

$CD4^+$ $CD45^+$ and express the retinoic acid receptor-related orphan nuclear receptor gamma t (RORγt) transcription factor (36). LTo cells seem to develop into follicular dendritic cells, which support B-cell responses, and fibroblast reticular cells that are required for T-cell survival and provide structural guidance to hematopoietic cells migrating through lymph nodes. A productive interaction between LTi and LTo cells depends mainly on the interaction between lymphotoxin (LT)-α1β2 on LTi cells and its receptor (LTβR) on LTo cells. After receiving signals through LTβR, LTo cells express adhesion molecules such as VCAM-1 and ICAM-1 and chemokines (CXCL13, CCL19 and CCL21) that drive the recruitment of T cells and B cells. No lymph nodes develop when these chemokines (or their receptors) are absent (36).

If the principles of lymphoid organogenesis, such as cross-talk between LTi and LTo cells, seem to apply to TLO formation, recent studies uncovered that the actors are not necessarily the same in SLO and TLO formation. For instance, it seems that vascular smooth muscle cells (VSMCs) could act as LTo cells in aortic TLO formation. Indeed, mouse aortic VSMCs produce the lymphorganogenic chemokines CXCL13 and CCL19 when stimulated *in vitro* simultaneously through TNFR-1 and LTβR (37). Furthermore, VSMCs located between lesions and TLOs are activated and produce CXCL13 and CCL21 (8). Finally, we recently showed in abdominal aortic aneurysms that VSMCs were the main source of CXCL13, CXCL16, CCL19, CCL20 and CCL21 chemokines, and that the production of these chemokines is triggered by the soluble mediators present in the intima, consistent with the fact that VSMCs can act as LTo cells (33).

The nature of the LTi cells in TLO formation, as well as of the molecules that they use to activate LTo cells, might also depend on the tissue where they form (35). We recently reported that M1 proinflammatory macrophages can act as LTi cells in atherosclerosis lymphoid neogenesis in mice (9). Indeed, M1 macrophages produced LT-α and TNF-α, two cytokines produced by LTi cells. Furthermore, M1 macrophages were able to induce an LTo profile in VSMCs. For example, in the presence of M1 macrophage-conditioned medium, VSMCs produced CXCL16, CCL19 and CCL20 chemokines and were able to induce TLO formation *in vivo* when seeded into scaffold implanted under the skin. However, the dialogue between M1 macrophages and VSMCs did not depend on LTβR and membrane-bound LT-α1β2, but rather on TNFR1 and TNFR2 and soluble LT-α3 and TNF-α. Accordingly, we demonstrated that TLO formation is independent of LTβR signaling *in vivo* in a model of aortic transplantation, whereas anti-TNF-α antibody treatment decreased the incidence and number of adventitial aortic TLOs in $ApoE^{-/-}$ mice. Interestingly, as the LTi potential of M1 macrophages mainly lies with the soluble factors that they produce, it is conceivable that other cells can act as LTi in TLO formation in atherosclerosis.

One crucial unanswered question about TLOs is whether they are beneficial or deleterious in atherosclerosis. TLOs, due to their proximity to diseased tissues, are thought to be rich in tissue-derived antigens, and therefore to be tailor-made sites for the adaptive immune response against those antigens to persist. Infection-induced TLOs are thought to be beneficial, as they participate in the generation of a protective immune response against the pathogen. On the contrary, in the context of autoimmune diseases such as rheumatoid arthritis or Sjögren syndrome, TLOs are pathogenic, as they sustain the differentiation of B cells into plasma cells producing high affinity autoantibodies recognizing disease-specific autoantigens (35). In transplantation, the situation is more complex, as TLOs have been observed both in the context of rejection (38) and tolerance (39,40), suggesting that they can sustain either a deleterious or a beneficial immune response. However, what drives the type of response generated in graft-associated TLOs remains unclear. Considering that the adaptive immune response can be either protective or pathogenic in atherosclerosis, it is difficult to predict the role of TLOs on disease progression. To complicate matters, it might be inaccurate to assume that the quality of the immune response is the same in TLOs and SLOs. In aortic allograft-associated TLOs, the local adaptive immune response in the adventitia displays unique characteristics in comparison to the immune response occurring in spleen and lymph nodes (41); this could also be the case in atherosclerosis. Further experiments will therefore be required to determine the function of TLOs in atherosclerosis and to design TLO-based therapeutic strategies in atherosclerosis.

LOOKING FOR A HAPPY ENDING THROUGH IMMUNE-BASED THERAPIES

Our understanding of the role of the adaptive immune system in atherosclerosis has improved considerably in the past few years, as described above, and should help in the design of new approaches targeting the immune system to prevent and/or treat atherosclerosis. Such approaches can aim either at boosting the protective immune response against atherosclerosis, by inducing antibodies against oxLDL, for instance, or at decreasing the proatherogenic immune response, which includes interfering with proatherogenic cytokines and targeting the deleterious NKT, Th1, Th17 and B2 cells.

The generation of a safe vaccine generating anti-oxLDL antibodies requires the identification of minimal oxLDL epitopes that can induce protection upon immunization. Peptide sequences derived from apolipoprotein B-100 (Apo B-100), a protein component of LDL, can induce atheroprotective antibodies (42), and an antibody directed against Apo B-100 has been tested in a phase II clinical trial. As another tactic, we chose to study the potential of phosphorylcholine (PC), a component of oxLDL phospholipids, as a target for atheroprotective immunization. We showed that PC immunization in

$ApoE^{-/-}$ mice boosted the levels of anti-PC and anti-oxLDL antibodies that were able to reduce foam cell formation from macrophages *in vitro* (25). Furthermore, PC-immunized $ApoE^{-/-}$ mice were protected from atherosclerosis, suggesting that it would be worth testing PC-based vaccine in humans.

Blocking proinflammatory cytokines, such as TNF-α, is an efficient treatment of some autoimmune diseases, such as rheumatoid arthritis. As TNF-α is proatherogenic in mice, TNF blockers could be used to decrease atherosclerosis in humans (43). However, such treatments increase the risk of infections, and, therefore, other approaches might be better in atherosclerosis. Interestingly, in line with the proatherogenic role of B2 cells, it has been suggested that rituximab (anti-CD20) can become a treatment in atherosclerosis (19,20). However, it would be important to verify that this treatment also depletes B cells from TLOs during atherosclerosis, considering that this is not the case in the context of allograft (34), and that aortic TLOs are sites of B-cell activation (29). Another strategy to impair B2 cell function would be to block their activation by Tfh cells. We demonstrated that treating mice with an anti-ICOSL antibody, which blocks Tfh development, decreases atherosclerosis (29); this suggests that anti-ICOSL treatment, which has been tested in phase I clinical trial in systemic lupus erythematosus, or other therapeutic strategies targeting the Tfh/GC B-cell axis, could be efficient in treating human atherosclerosis.

Strategies that would block effector T cells and/or enhance Treg responses also should be considered. Our data suggest that targeting the CD31 molecule would be a possible approach. CD31 is a transhomophilic tyrosine-based inhibitory motif receptor expressed by leukocytes, endothelial cells and platelets. When engaged, it decreases cell functions mediated by protein tyrosine kinases, such as TCR-mediated signal transduction in T cells (44,45). Furthermore, homophilic ligation of CD31 generates mutual repulsion of the interacting cells (45) and DC tolerization (46). Finally, we observed that patients with atherosclerotic abdominal aortic aneurysm lose CD31 at the surface of circulating T cells (47), suggesting that the pathologic immune response in atherosclerosis might be allowed by the loss of the inhibition induced by CD31 signaling. We therefore hypothesized that engaging CD31 would decrease T-cell activation, and possibly leukocyte recruitment to the lesions, in atherosclerosis. Indeed, $ApoE^{-/-}$ mice treated with a CD31 receptor globulin displayed reduced atherosclerotic lesions, together with less circulating activated T cells but more circulating $CD4^+$ Tregs (48). More recently, we demonstrated that a CD31-derived peptide that can engage CD31 signaling decreases aneurysm formation and plaque size in angiotensin-II-infused hypercholesterolemic $ApoE^{-/-}$ mice (49). Together, these data suggest that targeting the CD31 pathway could be a suitable therapeutic approach in atherosclerosis.

CONCLUSION

Studies on the function of the adaptive immune system in atherosclerosis have brought a better knowledge of the function of different cell subsets and revealed the importance to investigate not only the immune response locally in the intima and systemically in the circulation and SLOs, but also in the adventitia where TLOs develop and in which an adaptive immune response is mounted. Several questions remain unanswered, such as the role of TLOs in atherosclerosis. The challenge today is to translate what we have learned so far on the role of the different players of the adaptive immune system in atherosclerosis into immunotherapies that would prevent or treat atherosclerosis, decrease cardiovascular risks and help patients to live healthily ever after.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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