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B cell modulation in atherosclerosis

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General Introduction

Cardiovascular disease

Cardiovascular disease (CVD) encompasses all disorders associated with the heart and vascular system. It includes many serious disorders such as stroke, heart failure and myocardial infarction and as such is the leading cause of death globally¹. Furthermore, CVD also has a considerable financial burden in the Western society with for example a projected medical cost of 1.1 trillion dollar in the United States in 2035¹. The vast majority of CVD deaths can be attributed to coronary artery disease and stroke of which atherosclerosis is the main underlying cause. Atherosclerosis is a multifactorial disease with a long list of known risk factors. While some of these risk factors are fixed (i.e. gender, genetics, age), other risk factors, including smoking, excessive alcohol use, an unhealthy diet and physical inactivity are largely lifestyle-dependent. Greater understanding and improved management of these risk factors has resulted in a strong decline in CVD mortality since the 1970s. Nonetheless, the decline is stagnating in the last few years and it has been shown that risk factor control does not eliminate CVD^{1,2}. In fact, the most successful treatments so far have been plasma lipid lowering, which leads to a 30% reduction in relative risk of cardiovascular disease. In addition, it has been estimated that by 2035 approximately 45% of all United States adults will have some form of CVD due to the rapid increase in the number of old and obese people¹. These data clearly indicate that there is an urgent need for novel strategies to improve the prevention and treatment of atherosclerosis.

Atherosclerosis

Atherosclerosis is primarily characterized by the build-up of fatty material in the innermost layer of vessel walls. This process of arterial wall thickening was long considered as a normal and non-pathological consequence of aging. However, in the early 20th century several cardinal studies introduced the concept of atherosclerosis and the essential role of cholesterol in driving atherosclerosis³. Since then, the research community has devoted large efforts in elucidating the pathophysiology of atherosclerosis. This has led to a much greater understanding of the molecular and cellular intricacies in atherosclerosis, however this extended knowledge has not yet been translated into a definitive cure for atherosclerosis.

Development of atherosclerosis

Early lesions

Atherosclerosis is a chronic and slowly developing process. Lesion development often starts in an individual's teenage years which is typically without any clinical manifestation⁴. The formation of early lesions is closely linked with endothelial dysfunction, which primarily occurs in areas with disturbed local blood flow⁵. For this reason, lesions are often found within inner curvatures of arteries, where there is minimal shear stress, or at bifurcations, where blood flows oscillatory. The combination of local changes in blood flow with high levels of circulating cholesterol in the form of (very) low-density lipoproteins (VLDL and LDL) induces endothelial damage and/or activation⁶. This subsequently results in an increased permeability and expression of adhesion molecules on the endothelium. As a consequence, the presence of high amounts of plasma VLDL and LDL leads to passive diffusion of these lipoproteins through the now permeable endothelium⁷. In the vessel wall, lipoproteins undergo interactions with proteoglycans and are chemically modified⁸. The latter is considered a key step in the development of atherosclerosis, since modified LDL (e.g. oxidized LDL) further induces the endothelial cells to express adhesion molecules, chemokines and growth factors. The cumulative effect of the different processes is an increase of monocytes into the vessel wall⁹. In response to secreted growth factors, such as macrophage colony stimulating factor, monocytes

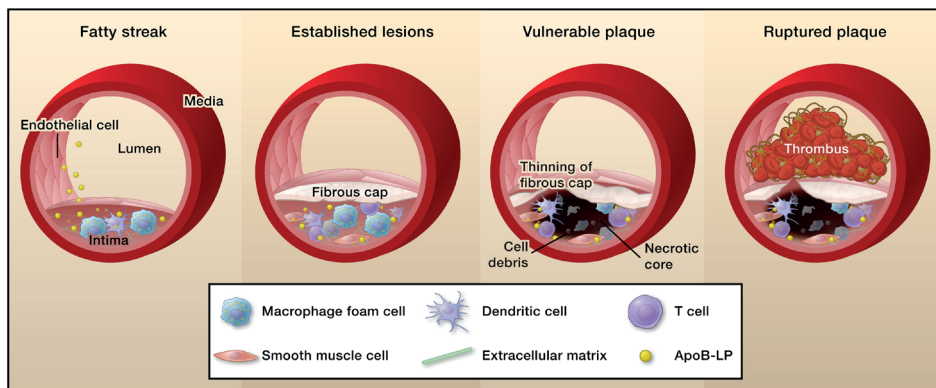


Figure 1. Development of atherosclerosis. Following endothelial damage, lipoproteins accumulate in the subendothelial space. Monocytes and macrophages are recruited to this early lesion and take up large amounts of cholesterol giving rise to fatty streaks. After failed clearance of the accumulated cholesterol and lipoproteins, more and more immune cells accumulate in the artery wall. Subsequently, smooth muscle cells are recruited which form a fibrous cap. The progression from an established lesion to a vulnerable plaque includes the thinning of the fibrous cap and the presence of large necrotic areas. Eventually, the cap can erode or rupture giving rise to a thrombus and the occurrence of acute clinical events. Adapted with permission from Moore KJ, Tabas I. *Cell* 2011;145:341–355.

locally differentiate into macrophages and upregulate their expression of scavenging receptors. Predominantly via these receptors, macrophages are efficient phagocytes and start clearing the vessel wall of the fatty material. This results in one of the hallmarks of early stage atherosclerosis; the presence of foam cells. Through the uptake of cholesterol esters, macrophages acquire a “foamy” look under the electron microscope and are thus called foam cells. The initial accumulation of foam cells results in a low-grade inflammation and a thin intimal cell layer known as fatty streaks¹⁰. This early immune response initially protects against further accumulation of immune cells and cholesterol. In fact, when combined with sufficient cholesterol lowering, these early lesions can still fully regress¹¹. Nonetheless, in most situations early lesions will eventually evolve into advanced lesions.

Advanced lesions

The formation of advanced lesions is primarily a result of an unrestrained immune response. Due to the constant accumulation of fatty material in the vessel wall, macrophages are unable to cope with the increased burden. This results in apoptosis of macrophages, which is a programmed cell death response¹². The effective removal of apoptotic cells by phagocytes occurs through a process called efferocytosis. Since macrophages are the predominant phagocytes in atherosclerotic lesions, the removal of apoptotic macrophages is largely dependent on the availability of viable macrophages. Hence, there is a very delicate balance between the number of apoptotic macrophages and the number of efferocytosis-mediating macrophages¹³. During atherosclerosis development, this balance is eventually disturbed due to the cholesterol overload. The failure of effective efferocytosis results in an accumulation of cellular debris, secondary necrosis and the leakage of cytoplasmic contents from dying macrophages, which all contributes to the formation of necrotic cores¹³. At this point, the ongoing lesional inflammation also results in the recruitment of other immune cells, such as neutrophils, dendritic cells and T cells, of which most of them perpetuate the ongoing inflammation. In addition, the local immune response also activates smooth muscle cells in the media, after which they migrate towards the intima. Smooth muscle cells produce extracellular matrix components such as collagen-rich fibers that stabilize the lesion^{14,15}. Additionally, some smooth muscle cells rapidly proliferate and migrate towards the lumen side of the lesion to form a protective fibrous cap^{16,17}. The combination of a thick fibrous cap and the presence of collagen is initially able to stabilize the lipid-rich necrotic areas and patients with well-stabilized lesions can be asymptomatic for years¹⁸. However, the next stage of atherosclerosis is destabilization of the lesion which is clinically the most important and obvious stage¹⁹. Several important aspects of lesion destabilization include thinning of the fibrous cap and lesion neovascularization. The precise mechanisms

behind thinning of the fibrous cap are not yet known, however it is believed that it is eventually due to increased apoptosis of the smooth muscle cells forming the cap¹⁸. The process of neovascularization is initiated due to the hypoxic areas in the lesion and destabilizes the lesion in several ways²⁰. Firstly, more immune cells are able to infiltrate the lesion via the newly formed blood vessels²¹. Secondly, the microvessels are often poorly formed which can result in intraplaque hemorrhages¹⁹. This latter results in the rapid accumulation of lesional erythrocytes and appears to be a critical contribution to lesion destabilization¹⁹.

Clinical events

Unrestrained progression of a lesion will eventually result in clinical manifestations²². Due to increased lesion volume, arteries can become stenotic, obstructing normal blood flow. Depending on the site of lesion, this may manifest itself in different symptoms such as chest pain in coronary artery stenosis²³. More acute cardiovascular events occur when unstable lesions erode or rupture, resulting in the formation of a thrombus. A thrombus can completely block blood flow and give rise to different cardiovascular complications, including stroke and heart attacks, depending on the tissue or organ affected²³. For these advanced stages, several surgical strategies are available. For example, via an endarterectomy the complete lesion is surgically removed²⁴. Other options include a vascular bypass operation²⁵ and balloon angioplasty to restore normal blood flow²⁶. However, all of these options have major drawbacks. The most prominent one is the increased possibility of restenosis due to the endothelial damage and local inflammation inflicted by the surgical procedures²⁷. Hence, prevention of atherosclerosis and the associated clinical events remains the most favorable option, however effective therapeutic options to do so are still scarce.

Experimental models of atherosclerosis

Due to the multifactorial character of atherosclerosis, the current *ex vivo* and *in vitro* models are insufficiently able to mimic the full spectrum of the disease pathology. Hence, to unravel the complex nature of atherosclerosis and to find novel therapeutic targets, the use of experimental animal models is still absolutely necessary. While zebra fish, rats, rabbits and non-human primates have been used, the most commonly used animal species for atherosclerosis research is the mouse²⁸. Mice are easy to genetically modify and breed, hence, they are ideally suited for experimental research. However, in contrast to humans, mice are very resilient against atherosclerosis and do not spontaneously develop this disease even when given a high

cholesterol diet. Thus two genetic knock-out models are now frequently used; apo-lipoprotein E-deficient (*ApoE*^{-/-}) mice and low-density lipoprotein receptor-deficient (*Ldlr*^{-/-}) mice. Both *ApoE*^{-/-} and *Ldlr*^{-/-} mice have a deficiency in lipid metabolism, which renders them susceptible to atherosclerosis development. While *ApoE*^{-/-} mice spontaneously develop lesions on a normal chow diet, *Ldlr*^{-/-} mice need to be fed a Western-type diet, which is high in fat and cholesterol content. The resulting atherosclerosis is relatively similar in these mice, however the nature of the atherogenic processes can substantially differ, which should be taken in consideration during experimental design²⁹.

Another model uses perivascular collars and induces site-specific lesions in the carotid artery. Silastic tubes are placed around the common carotid arteries which induces turbulent blood flow proximal to the collar. This disturbance in blood flow induces rapid lesions in combination with a Western-type diet in both *ApoE*^{-/-} and *Ldlr*^{-/-} mice³⁰.

A more novel approach is to use an adeno-associated virus with a gain-of-function for proprotein convertase subtilisin/kexin type 9 (PCSK9)³¹. Overexpression of PCSK9 effectively results in a largely similar phenotype as *Ldlr*^{-/-} mice, since PCSK9 targets the LDLR for degradation³². Since this requires only a single injection of virus, this novel method might eliminate the need for laborious breeding schemes to generate double knock-out mice.

Lipids in atherosclerosis

It is not surprising that the two most extensively used animal models are based on deficiencies in lipid metabolism, mimicking human familial hypercholesteremia. As discussed, the influence of cholesterol on atherosclerosis was already clearly established in the early 20th century. While cholesterol has an important physiological function in cell membrane structure³³ and steroid hormone production³⁴, excessive plasma levels have a strong correlation with atherosclerosis^{35,36}. Circulating levels of cholesterol originate in part from dietary cholesterol, however the great majority of cholesterol is endogenously produced in the liver³⁷. Since cholesterol is only minimally soluble in water, it is mainly transported by lipoproteins. Thus after it is released by the liver, cholesterol is mostly associated with VLDL. However, VLDL is also rich in triglycerides and its main function is to transfer triglycerides to tissues through the action of lipoprotein lipase. VLDL that is stripped of its triglycerides is a more dense particle (IDL) and is converted by hepatic lipase into LDL. Cells

obtain cholesterol through the LDLR, which binds circulating LDL and internalizes it through endocytosis. Subsequently, the cholesterol will be released internally and the LDLR is recycled and migrates back towards the cell surface. Circulating levels of lipoproteins are under strict control of hepatic expression of lipoprotein receptors (i.e. LDLR) which can bind both to ApoE in VLDL and to ApoB in LDL. Hence, genetic deficiency in either ApoE or LDLR results in unregulated levels of LDL and VLDL as seen in the experimental mouse models. Interestingly, while both VLDL and LDL are considered “bad cholesterol”, a third lipoprotein, HDL, is often considered as “good” cholesterol³⁸. HDL is involved in the reverse cholesterol transport from peripheral tissue to the liver. Not surprisingly, high plasma levels of HDL have been associated with decreased cardiovascular risks³⁹. Given the well-defined associations of VLDL/LDL and HDL with CVD, the lipid metabolism pathway has been an extensively investigated therapeutic target. Indeed, reducing VLDL/LDL by inhibition of hepatic cholesterol synthesis by statins has proven to significantly reduce cardiovascular risks. In contrast, therapeutic strategies to increase HDL have not been very successful. Quite recently, a next generation of lipid-lowering drugs that target PCSK9 have entered the market. As discussed, PCSK9 binds to the LDLR and targets it for degradation³². PCSK9 inhibitors effectively prevent this process, which results in higher numbers of hepatic LDLR, which enables them to clear more circulating cholesterol. Several phase III clinical trials have recently been performed demonstrating that PCSK9 inhibitors indeed decrease the serum cholesterol levels and the risk of cardiovascular events⁴⁰. Nonetheless, statins have been widely available for the last decades and we have seen a cardiovascular relative risk reduction of approximately 30%. The additional value of PCSK9 inhibitors has to be evaluated in the coming years, but it is clear that other processes than lipid metabolism contribute to atherosclerosis and CVD.

The immune system and atherosclerosis

In general, the immune system can be divided into two main arms; the innate and adaptive immune system. Cells of the innate immunity include neutrophils, monocytes, macrophages and dendritic cells. These cells quickly respond to pathogens and danger signals and act as a first-line of defense. Recognition of non-self antigens happens through pattern recognition receptors which recognize a broad range of antigens. Macrophages and dendritic cells are able to internalize and present these antigens to cells of the adaptive immune system; B and T cells. The adaptive immune system response is slower, however, the reaction is antigen-specific and results in immunological memory formation, which can result in long-lasting protection

against pathogens. This concept of memory formation is effectively harnessed in modern-day vaccination strategies.

The notion that the immune system is involved in atherosclerosis is not new. Rudolf Virchow already observed the inflammatory nature of atherosclerotic plaques in the 19th century⁴¹. His modern vision, however, was mostly ignored after the role of cholesterol and lipids was clearly established. For almost a century, atherosclerosis was mainly regarded as an entirely lipid-driven disease⁴¹. In the meantime, the general understanding of the immune system strongly increased and since the 1990s the contribution of the immune system to atherosclerosis gained a lot more attention. Nowadays, atherosclerosis is termed as a chronic inflammatory disease⁴² and the recently finished CANTOS trial unmistakably demonstrated that therapeutic intervention in the immune response can result in cardiovascular protection⁴³. However, there is a large variation in the individual contribution of immune cell subsets with both pro- and anti-atherogenic effects. Although the role of neutrophils⁴⁴, mast cells⁴⁵, eosinophils⁴⁶, NKT cells⁴⁷, NK cells⁴⁸, MDSCs⁴⁹ and ILCs⁵⁰ is outside the scope of this thesis, it should be noted that all of these cells influence atherosclerosis development. The role of monocytes, macrophages, dendritic cells, T cells and B cells will be further outlined below.

Monocytes

Monocytes are innate immune cells that develop in the bone marrow before being released into the circulation. They are relatively short-lived and either re-enter the bone-marrow or migrate into inflamed tissues after several days. Based on the expression pattern of CD14 and CD16, three human monocyte subsets have been determined; classical (CD14⁺⁺CD16⁻), non-classical (CD14⁺CD16⁺) and intermediate monocytes (CD14⁺⁺CD16⁺)⁵¹. Classical monocytes are the most abundant monocytes, infiltrate inflamed tissues and can differentiate into non-classical and intermediate monocytes⁵². Non-classical monocytes patrol healthy endothelium and rapidly respond to tissue injury⁵³. The intermediate monocytes represent the smallest population of monocytes and can secrete large amounts of TNF- α ⁵⁴. The gene expression profile of intermediate monocytes overlaps largely with that of classical monocytes and both can predict cardiovascular events^{55,56}. In mice two monocyte subsets have been identified, Ly-6C^{hi} and Ly-6C^{lo} which are similar to classical/intermediate monocytes and non-classical monocytes respectively⁵⁷. In general, it is believed that circulating monocytes represent a general population by which inflamed tissue are rapidly infiltrated⁵⁸. It has been shown that in response to high cholesterol levels, the number of monocytes strongly increases⁵⁹. This is due to both an increase in monocyte generation from precursors in the bone marrow as

well as increased recruitment of monocytes from the bone marrow⁶⁰. Additionally, in atherosclerosis the activated endothelium results in a rapid infiltration of Ly-6C^{hi} inflammatory monocytes into the vessel wall⁵⁹. Interestingly, this is one of the crucial steps in the initiation of atherosclerosis, while its contribution to established atherosclerosis seems minimal⁶¹. Nonetheless, the constant recruitment of Ly-6C^{hi} monocytes from the bone marrow and deposition into the lesion has been shown to greatly influence lesion size⁶². Particularly since the majority of these Ly-6C^{hi} monocytes differentiate into macrophages in the lesion⁵⁹.

Macrophages

As mentioned, macrophages are crucial in the development of atherosclerosis. Multiple studies have shown that the absence of macrophages significantly reduces atherosclerosis severity^{61,63,64}. However, the classical concept of macrophages as passive lipid collectors in the lesion has certainly been changed⁴¹. We now know that macrophages are very versatile immune cells that display a large degree of plasticity⁶⁵. Naïve macrophages (M0) are very sensitive for local inflammatory mediators and can polarize into a number of subsets. This is not a terminal differentiation, since changes in the micro-environment can stimulate macrophages to repolarize^{66,67}. This makes it difficult to describe the effects of each subset on atherosclerosis, since the macrophage phenotype is continuously shifting. Adding to the complexity, lesional macrophages share many markers with dendritic cells and smooth muscle cells^{68,69}. Nevertheless, mainly based on *in vitro* experiments, different classifications of macrophages have been made⁶⁵. In experimental systems, M1 macrophages can be generated by stimulation with LPS and IFN- γ . These macrophages initiate a strong pro-inflammatory response with the secretion of IL-1 β , IL-12 and TNF- α . Additionally they produce chemokines, such as MCP-1, to further increase the recruitment of monocytes. Hence, these M1 macrophages have a markedly pro-inflammatory response in order to kill pathogens upon infection and are thus termed classical macrophages. Non-classical macrophages include M2 macrophages, which can be polarized from M0 macrophages by IL-4 and IL-13. The immune response of M2 macrophages is in stark contrast to M1 macrophages, with the secretion of inflammation-resolving cytokines such as IL-10 and TGF- β . Pure M1 and M2 macrophage subsets might not be present in atherosclerotic lesions, but this classification has given a lot more insight in the differential function of macrophages in atherosclerosis⁶⁵. For instance, it is believed that in early lesions the majority of macrophages has a M2 phenotype which switches towards M1 during disease progression⁶⁶. In addition, M2 macrophages are found more frequently in plaques of asymptomatic patients, while M1 macrophages are more abundant in plaques from patients that suffered an acute ischemic attack⁷⁰. In functional assays,

M1 macrophages indeed have shown to secrete matrix metalloproteases which significantly add to the vulnerability of a lesion by breaking down extracellular matrix proteins⁷¹. They have also shown increased uptake of oxLDL and a debilitated ability for efferocytosis⁷¹. Hence, M1 macrophages seem to have a clear pro-atherogenic function. Contrary, M2 macrophages are superior phagocytes and have shown enhanced capability to clear apoptotic debris^{72,73}. Similar to M1 macrophages, M2 macrophages are found in lesions of mice⁶⁸ and humans, however, due to the production of anti-inflammatory cytokines M2 macrophages are believed to be atheroprotective. Also evidenced by the fact that polarization of macrophages towards a M2 phenotype by *Schistoma mansoni* infection reduces atherosclerosis⁷⁴. Besides M1 and M2 macrophages, other macrophages subsets, including M4⁷⁵ and Mox⁷⁶, with distinct functions have been described and the M1/M2 paradigm is now under debate⁷⁷. Indeed due to the plastic nature of macrophages, lesional macrophages can best be viewed as a dynamic range of phenotypes and functions⁶⁵. Despite the difficult nomenclature, the importance of macrophages in the pathophysiology of atherosclerosis is unmistakable.

Dendritic cells

A close relative of macrophages in terms of extracellular markers is the dendritic cell (DC). Since dendritic cells are professional antigen presenting cells (APCs) they function at the interface between the innate and adaptive immune system⁷⁸. They are present in all lymphoid and almost all non-lymphoid tissues. Similar to macrophages the distinction of different DC subsets has given rise to some debate^{79,80}. However, it is now understood that both conventional (cDC) and plasmacytoid (pDC) DCs are derived from a common DC progenitor while monocytes give rise to a different subset (moDCs)⁸⁰. The immature precursors patrol the periphery and lymphoid tissue in search for antigens. They pick up and process antigens in inflamed tissues while they differentiate into mature DCs. This is accompanied by a switch from a phagocytic phenotype towards an antigen-presenting phenotype with increased expression of costimulatory receptors such as CD80 and CD86 and CCR7. Subsequently they migrate and present antigens in nearby lymph nodes. The resulting antigen-specific immune response is largely dependent on the co-receptor and cytokine expression of the mature DC. DCs have the remarkable ability to skew an immune reaction towards both anti- and pro-inflammatory responses. The functional role of DCs in the pathogenesis of atherosclerosis is not fully clear⁸¹. They are present in the adventitia⁸², accumulate lipids and depletion of DCs in *Ldlr*^{-/-} mice has shown to attenuate atherosclerosis⁸³. In contrast, prolonged survival of DCs in either *Ldlr*^{-/-} or *apoE*^{-/-} mice did not result in accelerated atherosclerosis due to an atheroprotective decrease in serum cholesterol levels⁸⁴. Additionally, it has been demonstrated that

ex vivo oxLDL-pulsed DCs can be used to decrease lesion size⁸⁵. Due to the versatile nature of DCs it is difficult to pinpoint specific pro- or anti-atherosclerotic effects to different DC subsets⁸¹. Nonetheless, given their central role in the activation of T cells it remains of great interest to further dissect the effects of DC subsets in atherosclerosis.

CD8⁺ T cells

T cells originate from lymphoid progenitor cells in the bone marrow which undergo further development in the thymus. There they mature in either CD4⁺ or CD8⁺ T cells and leave the thymus for the periphery. CD8⁺ T cells or cytotoxic T cells recognize antigen on MHC-I molecules which is expressed on all nucleated cells. Under normal circumstances, cells continuously display cytosolic self-antigens in MHC-I molecules, which usually does not elicit an immune response. In an infected cell, however, non-self antigens are presented and recognized by CD8⁺ T cells. For an effective CD8⁺ T cell response cross-presentation of the non-self antigen by an APC is usually necessary⁸⁶, after which the CD8⁺ T cell kills the infected target cell. They also secrete large amounts of IFN- γ and TNF- α which further increases the local inflammation⁸⁷. The contribution of CD8⁺ T cells to atherosclerosis in humans appears primarily of atherogenic nature. They represent the majority of lesional lymphocytes⁸⁸ and a strong correlation between CD8⁺ T cells and coronary artery disease has been found previously^{89,90}. In contrast, the results in experimental mouse models do not fully support such a definitive conclusion. It was shown that CD8⁺ T cells strongly respond to a high fat diet as measured by their IFN- γ production⁹¹. In line with an proatherogenic contribution, it was further demonstrated that depletion of CD8⁺ T cells resulted in attenuated atherosclerosis while the adoptive transfer exacerbated disease severity⁹². On the other hand, mice lacking MHC-I molecules are unable to mount effective CD8⁺ T cell responses and show increased atherosclerosis⁹³. Other evidence supporting a protective role came from vaccination studies with an ApoB-100 epitope, which induced CD8⁺ T cell-mediated atheroprotection⁹⁴. An explanation for these discrepancies might lie in the presence of multiple distinct CD8⁺ T cell subsets⁹⁵. For instance, regulatory CD8⁺ T cells have recently been identified that specifically interacts with cells expressing the non-classical MHC-I molecule Qa-1⁹⁶. These regulatory CD8⁺ T cells have recently been demonstrated to inhibit specific CD4⁺ T cell response during atherosclerosis development which resulted in decreased lesion size⁹⁷. Others also showed that CD25⁺CD8⁺ T cells could confer atheroprotection⁹⁸. However the heterogeneity of CD8⁺ T cells includes more subsets than regulatory CD8⁺ T cells which have not yet been elucidated⁹⁵. Given their strong presence in human lesions, shedding more light on the contribution of CD8⁺ T cells in the pathogenesis of atherosclerosis remains necessary.

CD4⁺ T cells

In contrast to cytotoxic CD8⁺ T cells that require antigen to be presented on MHC-I molecules, CD4⁺ T helper cells recognize antigen presented by MHC-II molecules. Professional APCs such as DCs constitutively express MHC-II and it can also be induced on other cell types such as endothelial cells. CD4⁺ T cells regress from the thymus and migrate to secondary lymphoid organs, where they further mature into naïve CD4⁺ T cells. Besides antigen recognition, activation of a naïve CD4⁺ T cell requires the association with a stimulatory immune checkpoint protein. This drives autocrine IL-2 stimulation which induces T cell proliferation. Depending on the local inflammatory environment, unpolarized T helper (Th0) cells will differentiate into a specific Th cell subset. They are named Th cells since they help the activation or suppression of other immune cells. Each Th cell subset carries out a specific immune function and different Th cell subsets are often required in response to different pathogens. The influence of CD4⁺ T cells is therefore largely dependent on the Th subset.

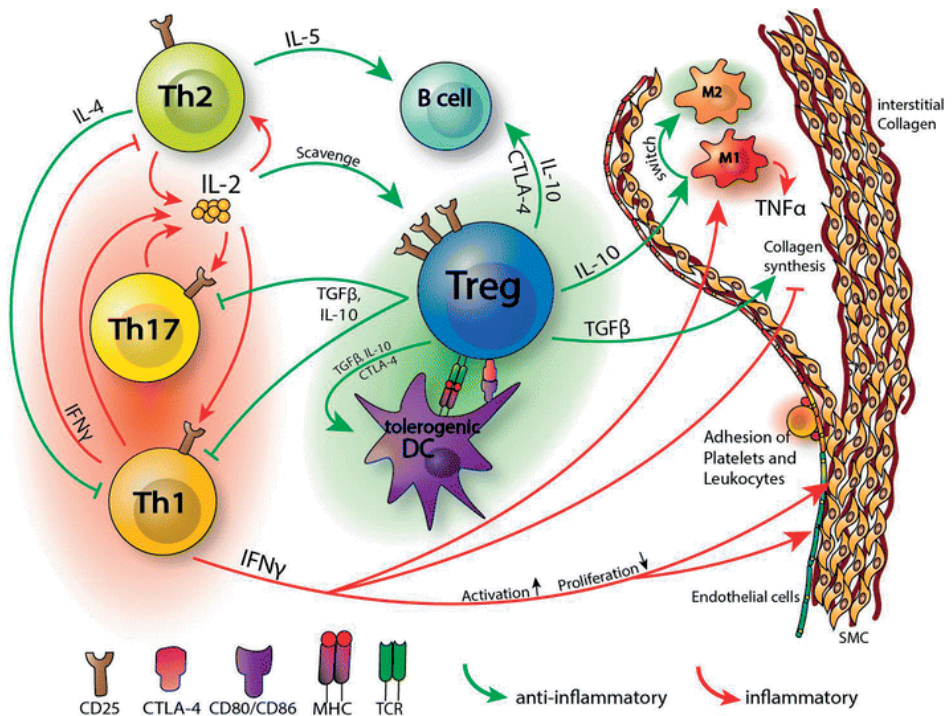


Figure 2. CD4⁺ T cell subsets and their key effector functions in atherosclerosis. Th1 cells primarily promote inflammation, while Th2 cells can have both pro- and anti-inflammatory responses. The role of Th17 cells is still controversial. Tregs dampen inflammation and have a well-defined protective effect on atherosclerosis. Adapted with permission from: Spitz *et al. Cell and Mol Li Sci* 73, no. 5 (1 March 2016): 901–22.

Th1 cells are induced in response to intracellular pathogens. They are induced by IFN γ , IL-12 and IL-18 which drives the expression of T-bet in CD4⁺ T cells. T-bet is the master regulator of Th1 cells and induces the production of pro-inflammatory cytokines such as IFN γ and TNF α . Th1 cells mainly activate macrophages, CD8⁺ T cells and the production of IgG2c by B cells, which aid the immune response against intracellular invaders. However, in atherosclerosis Th1 cells have a well-defined proatherogenic response. They are abundantly found in both murine and human lesions⁹⁹⁻¹⁰¹. Functional studies revealed that T-bet¹⁰² or IFN γ deficiency¹⁰³ resulted in markedly decreased atherosclerosis. Additionally, both IL-12 and IL-18 have prominent atherogenic roles via the induction of Th1 cells.

A second major CD4⁺ T cell subset is the Th2 cell. Th2 cells are the host immune defense in case of extracellular pathogens and their differentiation is promoted by IL-4 and IL-33. The key transcription factor in Th2 cells is Gata-3¹⁰⁴, which promotes IL-4, IL-5 and IL-13 expression¹⁰⁵. This results in the activation and degranulation of eosinophils¹⁰⁶ and mast cells¹⁰⁷ and the proliferation of B cells¹⁰⁸. Hence, Th2 cells mount an effective humoral immune response to battle extracellular pathogens. Their effect in the pathogenesis of atherosclerosis is, however, not so clear-cut. IL-5 has been shown to promote an atheroprotective B cell response¹⁰⁹ and IL-13 also reduced lesion size by skewing the macrophages towards a M2 phenotype¹¹⁰. However, IL-4 has been regarded as the hallmark Th2 cytokine and has demonstrated contradictory results. It has shown to strongly reduce the differentiation of Th1 cells¹¹¹, however it has no influence on atherosclerosis when administered¹¹². In fact, deficiency of IL-4 decreased lesion development, which is in line with a proatherogenic role¹¹³. Since IL-4, IL-5 and IL-13 are not exclusively produced by Th2 cells, it remains difficult to determine the exact contribution of Th2 cells to atherosclerosis without cell-specific KO models. Nonetheless, an imbalance between Th1 and Th2 cells seems to be associated with coronary artery disease^{114,115}.

A third subset is that of Th17 cells which seem to be important against certain fungal infections and are primarily characterized by their expression of ROR γ ^t¹¹⁶. This expression is induced by stimulation with TGF β and IL-6 and results primarily in the secretion of IL-17 and to a lesser extent of IL-21 and IL-22. Contradictory data of Th17 cells and IL-17 have been reported. Antibody-mediated inhibition of IL-17¹¹⁷ or IL-17 deficiency reduce lesion development¹¹⁸. Others also demonstrated that IL-17 deficiency did not affect atherosclerosis¹¹⁹. Additionally, it has been demonstrated that depletion of B cells resulted in an IL-17-mediated atheroprotection¹²⁰.

T follicular helper cells (Tfh) are a relatively new subset that have received a lot of attention in the last few years¹²¹. While Th1, Th2 and Th17 cells usually exit lymphoid tissues to migrate towards the site of inflammation, Tfh cells travel towards the B cell border to provide help for B cells¹²¹. They are characterized by expression of several markers such as Bcl-6, PD-1, CXCR5 and ICOS. They further mainly secrete IL-21 and IL-4 which strongly activates nearby B cells. The interaction between T and B cells in germinal centers will be discussed more thoroughly below. Nonetheless, several reports have investigated the effects of Tfh cells on atherosclerosis and for now have exclusively found an atherogenic effect^{97,122,123}.

A unique subset of CD4⁺ T cells that mainly inhibits other immune cells is the regulatory T cell (Treg). The importance of this subset mainly lies in the ability to maintain self-tolerance and to resolve inflammatory responses. TGF β plays an important role in the differentiation of Tregs¹²⁴, however weak interactions with the T cell receptor can also induce Tregs¹²⁵. Tregs can be identified by high expression of CD25 and FoxP3. CD25 is the IL-2 receptor and some of the immunosuppressive effects of Tregs are mediated through CD25¹²⁶. Moreover, FoxP3 directs the expression and secretion of IL-10 and TGF β which are strong anti-inflammatory cytokines¹²⁷. Given that most immune cells have a detrimental contribution to atherosclerosis, it is not surprising that the immune regulation of Tregs is clearly atheroprotective¹²⁸. Depletion of Tregs results in increased disease severity^{129,130}, whereas expansion of Tregs via an IL-2/anti-IL-2 complex or adoptive transfer of Tregs decrease lesion development^{131,132}. Given that low numbers of Tregs are associated with CVD and their well-defined effects in experimental models, they are a very promising target for the treatment of atherosclerosis (reviewed in¹²⁸).

B cells

Besides T cells, the other major component of the adaptive immune system is represented by B cells. In general there are two main populations of B cells that are distinct in origin and function, B1 and B2 cells.

B2 cells

B2 cells develop in the bone marrow from a common lymphoid progenitor which happens in sequential stages (Fig. 3). Early stages of B2 cell development involve the rearrangement of the heavy and light chain locus and once B cells express cell surface IgM they are considered as immature B cells (Fig. 3). Immature B cells are tested for self-reactivity and B cells with no strong reactivity are allowed to further mature in the blood and secondary lymphoid tissues. Immature B cells are also referred to as transitional B cells and it has been proposed that the maturation process

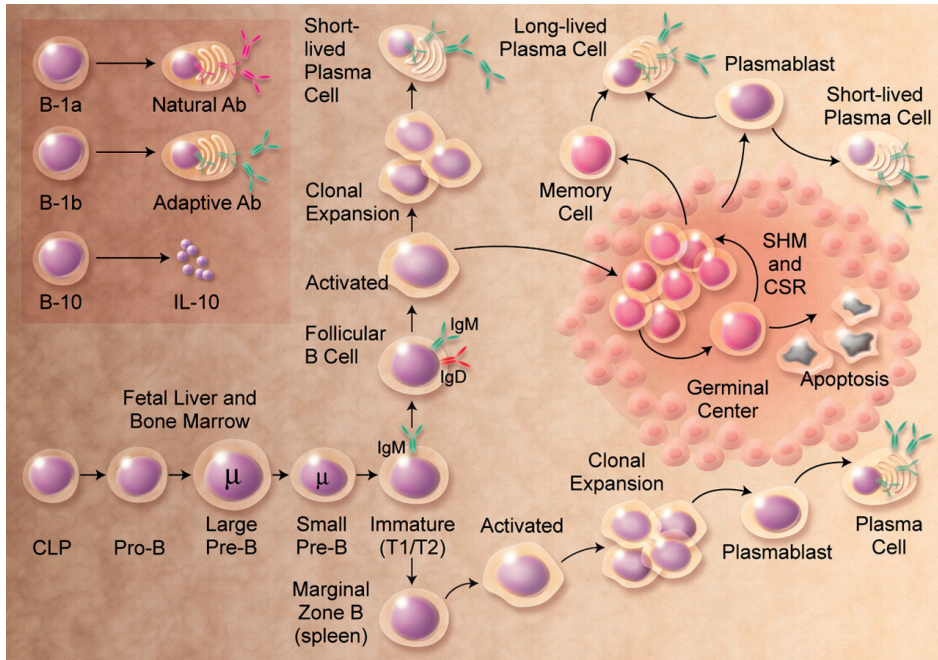


Figure 3. General overview of the development of different B cell lineages and subsets. Adapted with permission from: T.W. LeBien and T. F. Tedder. *Blood* 112, no. 5 (1 September 2008): 1570-80.

involves two consecutive stages with transitional type 1 and type 2 B cells¹³³. These transitional B cells can mature into either marginal zone (MZ) B cells or follicular (FO) B cells. The precise requirements and conditions for the differentiation into one of these subsets remain unknown. In fact, it is still under debate if this final selection happens in the periphery or is already predetermined during development in the bone marrow¹³⁴.

FO B cells represent the large majority of B cells and are characterized by high levels of IgD and CD23¹³⁵. They respond to T cell-dependent antigens and activation of FO B cells is initiated when it recognizes an antigen with its surface immunoglobulin. This initiates the upregulation of CCR7 which drives migration of the B cell towards the T-cell zones in lymphoid tissues. Subsequently the antigen is internalized and presented on MHC-II molecules to cognate CD4⁺ T cells. This triggers the activation of the CD4⁺ T cell to express extracellular markers and cytokines to further stimulate the B cell. This includes CD40 ligand expression which is a particular important CD4⁺ T cell effector molecule which binds to CD40 on the B cell. This interaction is important for almost all phases in B cell activation, including proliferation, class-switching and somatic hypermutation¹³⁶. After several rounds of proliferation, some

B cells differentiate into antibody-producing plasmablasts. These cells are often considered as immature plasma cells and secrete antibodies for some days after which they either die or further differentiate into plasma cells. The generation of most plasma cells, however, is through a longer route in germinal centers. Proliferating B cells represent the majority of immune cells in the germinal center, but Tfh cells and follicular dendritic cells are also crucial components. In the germinal center, B cells receive stimuli from Tfh cells to undergo isotype switching, which is a process that changes the constant-region of the immunoglobulin heavy-chain. This process is highly dependent on the cytokine production by T cells and follicular dendritic cells. For instance, Th2 cells are potent B cell activators and primarily induce IgG1 and IgE production. On the other hand, Th1 cells only minimally activate B cells but skew antibody production towards an IgG2 isotype¹³⁷. Subsequently, they undergo somatic hypermutation which introduces point-mutations in the variable immunoglobulin region which enables affinity maturation. In this process, B cells compete for survival signals from Tfh cells which promotes clones with increased antigen-affinity. Finally, B cells receive an as of yet unknown terminal differentiation signal which induces BLIMP-1 expression. BLIMP-1 effectively suppresses proliferation and drives the generation of plasma cells¹³⁸. Plasma cells secrete large amounts of antibodies and downregulate CXCR5 and CCR7 which permits them to leave the germinal center and migrate towards peripheral tissue¹³⁹. Other B cells turn into memory B cells which remain dormant until antigen is reintroduced. The current vision on this germinal center reaction and follicular B2 cells mainly portrays an atherogenic role which will be further discussed in further chapters.

The second major B2 cell subset is that of MZ B cells, which follow a completely different activation pathway. They are easily distinguished from FO B cells due to their high expression of CD21 and IgM and low levels of CD23. MZ B cells are uniquely located at the blood interface in the spleen and rapidly respond to blood-borne pathogens¹⁴⁰. Although they are able to form germinal centers in response to T cell-dependent antigens, these are often poorly organized¹⁴¹. In contrast, they usually recognize T cell-independent antigens after which they rapidly produce polyreactive IgM antibodies. It has recently been shown that MZ B cells accumulate during atherosclerosis development. However, removal of MZ B cell aggravated atherosclerosis suggesting that the increase in MZ B is a protective response¹²³.

B1 cells

B1 cells are a distinct subset of B cells that are derived from precursors in the fetal liver¹⁴². In adult life, B1 cells mainly reside in the peritoneal cavity and their population is primarily dependent on self-renewal instead of recruitment of new cells from

the bone marrow. Although they are derived from a different origin, B1 cells share many similarities with MZ B cells. Together with MZ B cells, the B1 cells can be regarded as innate-like B cells that are placed at the interface between the innate and adaptive immune response¹⁴³. B1 cells can quickly respond to T cell-independent antigens by production of IgM. Additionally, they also spontaneously secrete IgM antibodies in absence of antigen with a broad specificity. These natural IgM antibodies play a crucial role as a first line of defense in the humoral immunity¹⁴⁴. B1 cells are able to protect against atherosclerosis, which will be further described in the next chapter.

Thesis outline

It is clear from all provided evidence that the immune system has a considerable impact on atherosclerosis. While the contribution of most immune cells has been investigated comprehensively in the last decade, the number of reports on B cells and atherosclerosis is still limited. More specifically, B cells have long been exclusively regarded as antibody-producing cells. However, we nowadays know that B cells are very versatile and dynamic immune cells with a broad range of actions. The resulting effect of these actions on atherosclerosis are only minimally investigated. Hence, the overall aim of this thesis is to modulate the B cell response in atherosclerosis and gain more insight into the antibody-independent roles of B cells during atherosclerosis. In **Chapter 2** we reviewed the addition of novel subsets to the B cell family and their known roles in atherosclerosis. We have described that besides B1 and B2 cells, more and more B cell subsets have been identified which are minimally investigated in the context of atherosclerosis. In **Chapter 3** we aimed to elucidate the role of IL-10-secreting B cells in atherosclerosis. We show that these regulatory B cells potently inhibit the immune system during atherosclerosis development. However, we also show a hitherto unknown effect of B cell adoptive transfer on cholesterol homeostasis which could have masked the effects of IL-10 producing B cells on atherosclerosis. We further investigated IL-10 producing B cells using mice deficient in TIM-1 signaling (TIM-1^{Δmucin}) in **Chapter 4**. We show that these mice are deficient in IL-10⁺ B cells and display increased lesion development compared to wild-type mice. Furthermore, these mice showed a specific reduction in the Th2 response, which potentially contributed to the increased lesion development in TIM-1^{Δmucin} mice. In **Chapter 5** we have described a different regulatory B cell subset expressing high levels of PD-L1. We effectively generated this subset by *ex vivo* stimulation of B cells with IFN- γ . We further show that this subset is able to inhibit Tfh cells *in vitro* and *in vivo*. Additionally, adoptive transfer of these IFN γ -stimulated B cells resulted

in decreased lesion formation. **Chapter 6** describes role of the immune-checkpoint inhibitor BTLA in atherosclerosis. We first demonstrate that BTLA is mainly expressed on B cells in tissue from atherosclerotic patients and *Ldlr*^{-/-} mice. Next, with the use of an agonistic antibody for BTLA in atherosclerosis, we demonstrate that this antibody selectively reduced follicular B cells, while other B cells remained unaffected. This resulted in diminished CD4⁺ T cell activation and an increase in the number of Tregs. We further revealed that treatment with the BTLA antibody reduced lesion development, besides also inducing lesion stabilization in established lesions. Concluding remarks and future perspectives on all results described in this thesis are found in **Chapter 7**.

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