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B cell modulation in atherosclerosis

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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 globally[1]. 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 2035[1]. 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 people[1]. 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 atherosclerosis[3]. 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 manifestation[4]. The formation of early lesions is closely linked with endothelial dysfunction, which primarily occurs in areas with disturbed local blood flow[5]. 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 activation[6]. 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 endothelium[7]. In the vessel wall, lipoproteins undergo interactions with proteoglycans and are chemically modified[8]. 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 wall[9]. In response to secreted growth factors, such as macrophage colony stimulating factor, monocytes

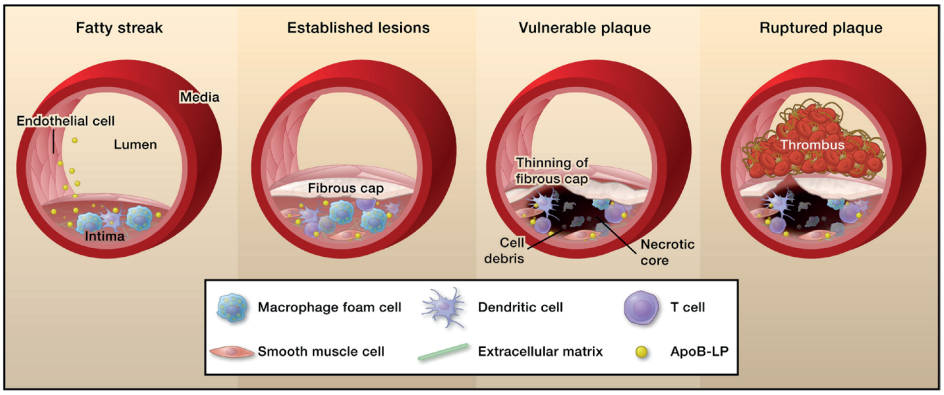


Figure 1. Development of atherosclerosis. Following endothelial damage, lipoproteins accumulate in the subendothelial space. Monocytes and macrophages are recruited to this early lesion and take up large amounts of cholesterol giving rise to fatty streaks. After failed clearance of the accumulated cholesterol 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 streaks[10]. 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 regress[11]. 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 response[12]. 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 macrophages[13]. 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 cores[13]. 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 years[18]. However, the next stage of atherosclerosis is destabilization of the lesion which is clinically the most important and obvious stage[19]. 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 cap[18]. The process of neovascularization is initiated due to the hypoxic areas in the lesion and destabilizes the lesion in several ways[20]. Firstly, more immune cells are able to infiltrate the lesion via the newly formed blood vessels[21]. Secondly, the microvessels are often poorly formed which can result in intraplaque hemorrhages[19]. This latter results in the rapid accumulation of lesional erythrocytes and appears to be a critical contribution to lesion destabilization[19].

Clinical events

Unrestrained progression of a lesion will eventually result in clinical manifestations[22]. 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 stenosis[23]. 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 affected[23]. For these advanced stages, several surgical strategies are available. For example, via an endarterectomy the complete lesion is surgically removed [24]. Other options include a vascular bypass operation[25] and balloon angioplasty to restore normal blood flow[26]. 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 procedures[27]. 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 mouse[28]. 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; apolipoprotein 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 design[29].

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*$^{-/-}$ mice[30].

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 degradation[32]. 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 structure[33] and steroid hormone production[34], 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 liver[37]. 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" cholesterol[38]. 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 risks[39]. 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 degradation[32]. 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 events[40]. 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 century[41]. 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 disease[41]. 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 disease[42] and the recently finished CANTOS trial unmistakably demonstrated that therapeutic intervention in the immune response can result in cardiovascular protection[43]. 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 neutrophils[44], mast cells[45], eosinophils[46], NKT cells[47], NK cells[48], MDSCs[49] and ILCs[50] 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 monocytes[52]. Non-classical monocytes patrol healthy endothelium and rapidly respond to tissue injury[53]. 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 respectively[57]. In general, it is believed that circulating monocytes represent a general population by which inflamed tissue are rapidly infiltrated[58]. It has been shown that in response to high cholesterol levels, the number of monocytes strongly increases[59]. 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 marrow[60]. Additionally, in atherosclerosis the activated endothelium results in a rapid infiltration of Ly-6C^{hi} inflammatory monocytes into the vessel wall[59]. Interestingly, this is one of the crucial steps in the initiation of atherosclerosis, while its contribution to established atherosclerosis seems minimal[61]. 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 size[62]. Particularly since the majority of these Ly-6C^{hi} monocytes differentiate into macrophages in the lesion[59].

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 changed[41]. We now know that macrophages are very versatile immune cells that display a large degree of plasticity[65]. 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 made[65]. 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-1β, 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 atherosclerosis[65]. For instance, it is believed that in early lesions the majority of macrophages has a M2 phenotype which switches towards M1 during disease progression[66]. 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 attack[70]. 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 proteins[71]. They have also shown increased uptake of oxLDL and a debilitated ability for efferocytosis[71]. 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 mice[68] 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 atherosclerosis[74]. Besides M1 and M2 macrophages, other macrophages subsets, including M4[75] and Mox[76], with distinct functions have been described and the M1/M2 paradigm is now under debate[77]. Indeed due to the plastic nature of macrophages, lesional macrophages can best be viewed as a dynamic range of phenotypes and functions[65]. 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 system[78]. 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 clear[81]. They are present in the adventitia[82], accumulate lipids and depletion of DCs in *Ldlr*$^{-/-}$ *mice* has shown to attenuate atherosclerosis[83]. 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 levels[84]. Additionally, it has been demonstrated that

ex vivo oxLDL-pulsed DCs can be used to decrease lesion size[85]. Due to the versatile nature of DCs it is difficult to pinpoint specific pro- or anti-atherosclerotic effects to different DC subsets[81]. 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 necessary[86], 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 inflammation[87]. The contribution of $CD8^+$ T cells to atherosclerosis in humans appears primarily of atherogenic nature. They represent the majority of lesional lymphocytes[88] 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 production[91]. 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 severity[92]. On the other hand, mice lacking MHC-I molecules are unable to mount effective $CD8^+$ T cell responses and show increased atherosclerosis[93]. Other evidence supporting a protective role came from vaccination studies with an ApoB-100 epitope, which induced $CD8^+$ T cell-mediated atheroprotection[94]. An explanation for these discrepancies might lie in the presence of multiple distinct $CD8^+$ T cell subsets[95]. For instance, regulatory $CD8^+$ T cells have recently been identified that specifically interacts with cells expressing the non-classical MHC-I molecule Qa-1[96]. These regulatory $CD8^+$ T cells have recently been demonstrated to inhibit specific $CD4^+$ T cell response during atherosclerosis development which resulted in decreased lesion size[97]. Others also showed that $CD25^+CD8^+$ T cells could confer atheroprotection[98]. However the heterogeneity of $CD8^+$ T cells includes more subsets than regulatory $CD8^+$ T cells which have not yet been elucidated[95]. 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 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.

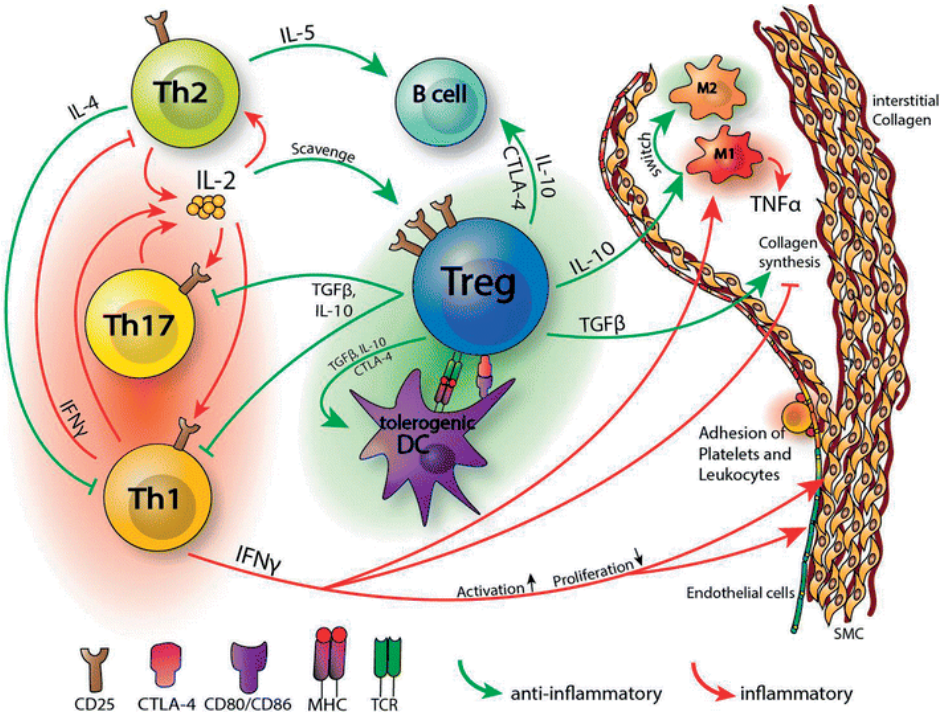


Figure 2. CD4$^+$ T cell subsets and their key effector functions in atherosclerosis. Th1 cells primarily promote inflammation, while Th2 cells can have both pro- and anti-inflammatory responses. The role of Th17 cells is still controversial. Tregs dampen inflammation and have a well-defined protective 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 IFNγ, 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 IFNγ and TNFα. 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 lesions[99–101]. Functional studies revealed that T-bet[102] or IFNγ deficiency[103] 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-3[104], which promotes IL-4, IL-5 and IL-13 expression[105]. This results in the activation and degranulation of eosinophils[106] and mast cells[107] and the proliferation of B cells[108]. 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 response[109] and IL-13 also reduced lesion size by skewing the macrophages towards a M2 phenotype[110]. 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 cells[111], however it has no influence on atherosclerosis when administered[112]. In fact, deficiency of IL-4 decreased lesion development, which is in line with a proatherogenic role[113]. 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 RORγt[116]. This expression is induced by stimulation with TGFβ 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-17[117] or IL-17 deficiency reduce lesion development[118]. Others also demonstrated that IL-17 deficiency did not affect atherosclerosis[119]. Additionally, it has been demonstrated that depletion of B cells resulted in an IL-17-mediated atheroprotection[120].

T follicular helper cells (Tfh) are a relatively new subset that have received a lot of attention in the last few years[121]. 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 cells[121]. 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. TGFβ plays an important role in the differentiation of Tregs[124], however weak interactions with the T cell receptor can also induce Tregs[125]. 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 CD25[126]. Moreover, FoxP3 directs the expression and secretion of IL-10 and TGFβ which are strong anti-inflammatory cytokines[127]. Given that most immune cells have a detrimental contribution to atherosclerosis, it is not surprising that the immune regulation of Tregs is clearly atheroprotective[128]. 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 in[128]).

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

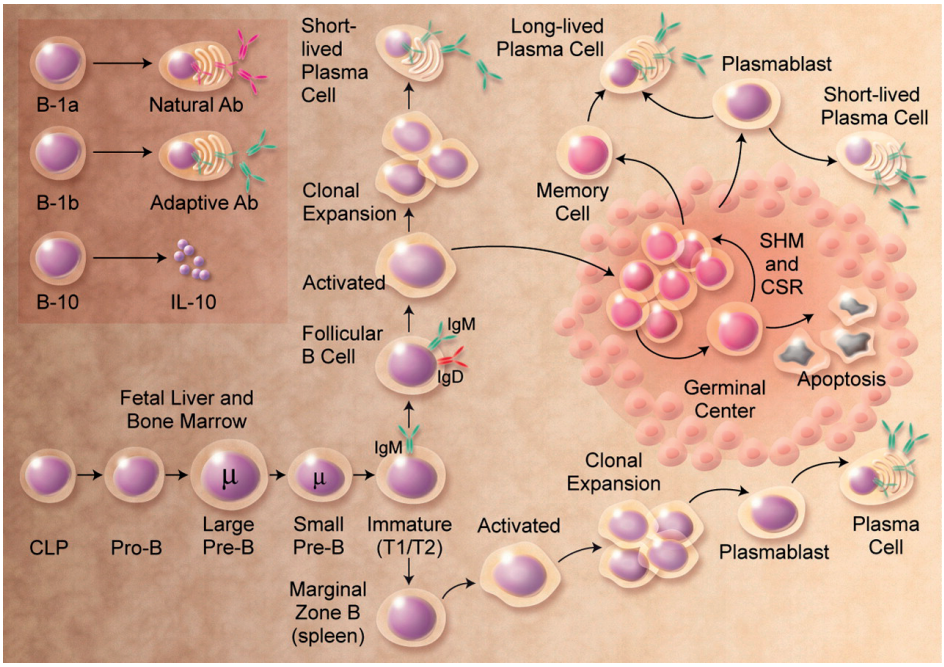


Figure 3. General overview of different the development of different B cell lineages and subsets. Adapted with permission from: *T.W. LeBien and T. F. Tedder. Blood 112, no. 5 (1 September 2008): 1570–80.*

involves two consecutive stages with transitional type 1 and type 2 B cells[133]. 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 marrow[134].

FO B cells represent the large majority of B cells and are characterized by high levels of IgD and CD23[135]. 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 hypermutation[136]. 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 isotype[137]. 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 cells[138]. Plasma cells secrete large amounts of antibodies and downregulate CXCR5 and CCR7 which permits them to leave the germinal center and migrate towards peripheral tissue[139]. 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 pathogens[140]. Although they are able to form germinal centers in response to T cell-dependent antigens, these are often poorly organized[141]. 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 response[123].

B1 cells

B1 cells are a distinct subset of B cells that are derived from precursors in the fetal liver[142]. 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 response[143]. 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 immunity[144]. 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$^{\Delta mucin}$) 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$^{\Delta mucin}$ 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 IFNγ-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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Novel B cell subsets in atherosclerosis

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Abstract

Purpose of review

The number of deaths associated with cardiovascular disease remains high, despite great advances in treating the associated high levels of cholesterol. The main underlying pathology of cardiovascular disease is atherosclerosis, which is recognized as a chronic autoimmune-like inflammatory disease. Hence, there is a pressing need to shed light on the immune pathways associated with atherosclerosis. B cells have long been thought to have a general protective effect in atherosclerosis. However, findings in the last decade have challenged this paradigm, showing that it is crucial to differentiate between the various B cell subsets when assessing their effect on atherosclerosis.

Recent findings

It has become increasingly recognized that B cells can have significant effects on the immune system independent of antibody production. The understanding that B cells form a major source of cytokines and can directly influence T cell responses via surface markers, have led to the identification of novel B cell subsets. These subsets are important modulators of auto-immune disorders but have not yet been fully investigated in atherosclerosis.

Summary

Here we review the current known roles of B cell subsets and the putative effects of recently identified B cells on atherosclerosis.

Introduction

Atherosclerosis has long been established as a chronic autoimmune-like inflammatory disease. Substantial work in the last years has identified a broad spectrum of immune cells and immunomodulatory checkpoints that can have a significant contribution to the onset and/or progression of atherosclerosis. Immune cells such as macrophages, monocytes, neutrophils, dendritic cells and T cells have been extensively investigated and as such their impact on atherosclerosis has been illuminated to a great extent.

In addition, already in 2002 it was shown that B cells can also have a substantial impact on the pathophysiology of atherosclerosis[1,2]. Two independent studies showed a general atheroprotective effect on atherosclerosis. Since then, the number of studies on B cells in atherosclerosis has somewhat waned compared to the number of papers examining T cells. However, the discovery that different B cell subsets can have opposing effects on atherosclerosis has led to a resurge in interest in B cells and atherosclerosis in recent years. As a result, the two main B cell lineages and their effects on atherosclerosis are now understood to a certain extent. In contrast, novel B cell subsets have been recently identified which could have great potential in atherosclerosis and have not yet been fully explored.

In this review we briefly discuss the current understanding of the role of classical B cells and the putative contribution of novel B cell subtypes to atherosclerosis.

B1 and B2 and antibody production

Broadly, B cells can be divided into two lineages that differ greatly in ontogeny, function, location and surface marker expression: B1 and B2 cells (Fig. 1). B1 cells are the dominant type of B cell in neonates and originate mainly from fetal liver and omentum. In adult life, they reside mostly in pleural and peritoneal cavities and respond to self- and T-independent antigens by secreting polyreactive natural IgM antibodies. Based on the expression of the surface marker CD5, B1 cells can be further subdivided in B1a and B1b cells. Alternatively, the large majority of B cells in the adult is comprised of B2 cells that include the conventional follicular B cells and marginal zone B cells. The latter are often considered as part of the innate immune system, since they rapidly respond to blood-borne antigens, including bacterial cell wall components and self-antigens, with polyreactive antibodies. However, the largest population of splenic B cells consists of follicular B cells that undergo isotype

switching in germinal centers in response to T-cell dependent antigens and either give rise to memory B cells or antibody secreting plasma cells.

In 2002, two groups provided the first compelling evidence on the modulatory properties of B cells in atherosclerosis[1,2]. Initially, these two corroborating studies led to the idea of B cells having an overall atheroprotective effect. However, later studies

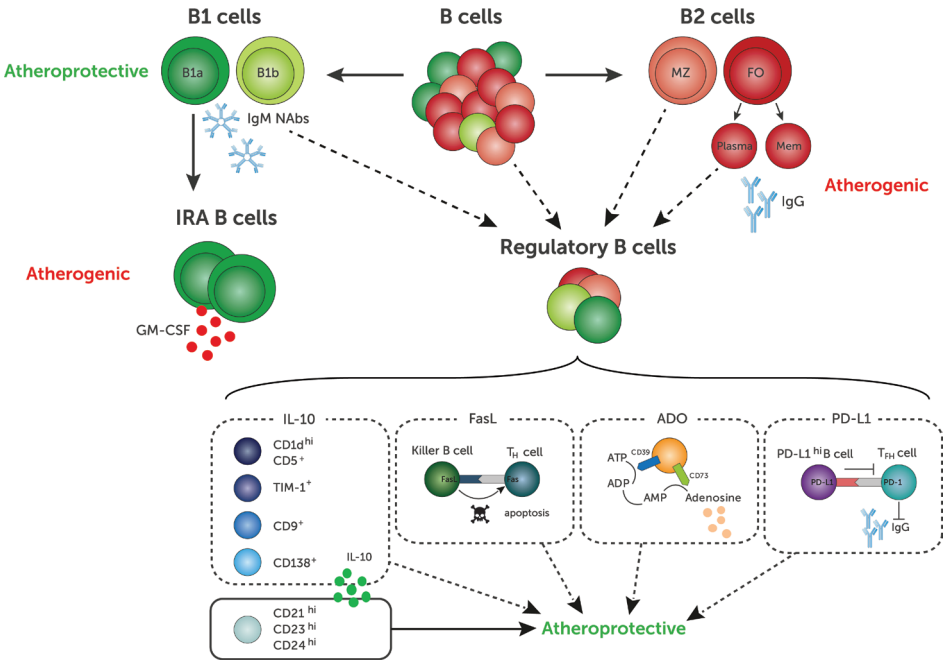


Figure 1. Overview of the main B cell lineages and novel B cell subtypes with their known and putative effects on atherosclerosis. B cells can be divided into two main lineages; B1 and B2 cells. B1 cells can be further divided into B1b and B1a B cells that both attenuate atherosclerosis by the secretion of natural IgM antibodies (IgM NAbs). IRA B cells are derived from B1a cells and are characterized by GM-CSF production by which they promote inflammation in the spleen, which results in increased atherosclerosis. Conventional B2 cells, including marginal zone (MZ) and follicular (FO) B cells, are generally believed to have an atherogenic effect, which may be caused by their IgG secretion. Regulatory B cells (B_{regs}) negatively influence the immune response and hence could have potential atheroprotective effects. They share markers with many other B cell subsets and it remains unknown if they develop from a specific type of B cell or if all B cells can become regulatory when provided with the right immunological context. The majority of B_{regs} are dependent on IL10 production and many different markers have been ascribed to these cells, such as $CD1d^{hi}CD5^{+}$, $TIM\text{-}1^{+}$, $CD9^{+}$, $CD138^{+}$ and $CD21^{hi}CD23^{hi}CD24^{hi}$ B cells. Only the latter have already been reported to protect against atherosclerosis. Killer B cells can induce apoptosis in T_H cells by their expression of FASL. B cells expressing CD39 and CD73 can drive the purinergic response from the ATP to ADP and adenosine that is associated with atheroprotection. B cells expressing high levels of PD-L1 can inhibit the T_{FH}-germinal center axis and decrease the secretion of high-affinity IgG antibodies. *Dotted lines/arrows depict not yet determined roles in atherosclerosis.

challenged this paradigm by pointing out major differences between B1 and B2 cells in modulating atherosclerosis[3–5]. Currently, the evidence for a protective function of B1a cells is firmly established[5–8]. There is a widespread agreement that B1 cells protect against atherosclerosis due to the production of natural IgM antibodies that induce the clearance of oxidized low-density lipoproteins (oxLDL) and apoptotic cells[9–11]. In addition, it was recently shown that B1b cells contain similar atheroprotective characteristics by secreting natural antibodies[12]. On the other hand, there is an abundance of data claiming an atherogenic effect of B2 cells[3,4,13,14]. The most important evidence for this statement is provided by depletion studies, which show that selective depletion of B2 cells limits the development of atherosclerosis[3,4,13,14]. One reason for this is that the antibodies secreted by B2 cells are usually of the IgG isotype which are, in contrast to IgM antibodies, often associated with aggravated atherosclerosis. However, the understanding about the effects of different isoforms of IgG is still very limited[15]. In addition, the exact influence of B2 cells on atherosclerosis remains debatable, since conflicting evidence shows that adoptively transferred B2 cells can also reduce lesion size[16,17]. The main difference between these apparent contradictory studies is the number and genotype of adoptively transferred B cells, $5x10^6$ B cells from C57BL/6J (BL/6) mice versus $30x10^6$ B cells from apolipoprotein E knockout (*apoE*$^{-/-}$) mice[4,16]. This discrepancy points out the importance of taking into consideration that not all B2 cells are similar and that the source and number of cells used can have a major influence on the development of atherosclerosis.

In recent years, it has been shown that within the B1 and B2 lineages additional B cell subsets can be identified (Fig. 1). While most papers discussing B1 and B2 cells often emphasize their antibody producing capacities, the majority of these novel B cell subsets are either identified based on secretion of specific cytokines or surface marker expression. These B cells can modulate immune responses by altering the cytokine network or directly altering T cell responses via co-receptor expression. These emerging concepts play major roles in auto-immune disorders, but are not yet fully appreciated in the context of atherosclerosis.

IL-10 dependent regulatory B cells

One major group of B cells that has seen a lot of attention in the last decade is that of regulatory B cells ($B_{regs,}$ Fig. 1). In general, B cells that negatively modulate the immune response are considered B_{regs} and the majority of these B cells depend in one way or another on the secretion of interleukin-10 (IL-10)[18]. IL-10 is an extensively studied

anti-inflammatory cytokine with well-known anti-atherogenic properties[19–21]. It is primarily secreted by macrophages and T cells and protects against atherosclerosis in several ways, including inhibition of macrophage and T cell activation but it also has impact on lipid metabolism[21,22]. It is now known that B cells are also capable in regulating auto-immune disorders via secretion of IL-10[23], but this concept has only recently been investigated in atherosclerosis[24,25]. Using a chimeric system to generate *Ldlr*$^{-/-}$ mice with a B cell specific deficiency in IL-10, Sage et al. showed that a loss of B cell-derived IL10 unexpectedly did not result in a significant effect on plaque size[24]. A second study, however, showed that B-cell derived IL-10 is actually capable of reducing atherosclerosis development in *apoE*$^{-/-}$ mice[25]. Strom et al. reported a significant increase in IL10^{+} B cells in the lymph nodes of female *apoE*$^{-/-}$ mice compared to BL/6 mice. Subsequent adoptive transfer of these lymph node B cells into *apoE*$^{-/-}$ mice protected them against collar-induced neo-intima formation. This effect was IL10-dependent since concurrent injection with an IL-10 neutralizing antibody abrogated the effects. Similarly, adoptive transfer of IL-10$^{-/-}$ B cells failed to protect against atherosclerosis[25]. The apparent discrepancy between these two studies can in part be explained by their different experimental approaches (e.g. *apoE*$^{-/-}$ vs. *Ldlr*$^{-/-}$ or chow diet vs. high-fat diet). However, another possibility is that IL10-producing B cells or the CD21^{hi}CD23^{hi}CD24^{hi} B cell population as used by Strom et al. does not exclusively secrete IL-10. Two recent papers elegantly show that IL-35 is able to induce IL-10-producing B cells which in turn also secrete large amounts of IL-35 that can suppress autoimmune disease[26,27]. As suggested by others, there could be a complex interplay between IL-35 and IL-10 in mediating immunosuppression[28]. Thus, silencing only one of these cytokines could lead to compensatory mechanisms resulting in upregulation of the cytokine by other cell types or the induction of other anti-inflammatory cytokines. For instance, in the aorta of mice deficient for B cell derived IL-10 a significant increase in IL-10 gene expression was found, while IL-35 or transforming growth factor β were not measured[24]. Moreover, the IL-10 blocking antibody used by Strom et al. did not neutralize the decrease in lesional macrophages, again displaying only a partial response of IL-10-mediated immune suppression[25].

Adding to the complexity is the lack of a unifying surface marker or transcription factor to identify IL-10 producing B cells. Currently, there is a growing collection of literature that identifies different B cell subsets in which IL10-producing B cells are enriched[29–33]. Many of these subsets are frequently used as biomarker for IL10-producing B cells or for adoptive transfer experiments. Since these subsets differ largely in their surface marker expression and also in the actual number of IL-10 producing B cells, the research into the exact contribution of B cell derived IL-10 to

atherosclerosis and other auto-immune disorders remains a challenge. It will thus be interesting to examine the effects of adoptive transfer studies using pure IL-10 producing B cells. In addition, studies that shed light on the complex network of multiple immune-suppressive B cell derived cytokines are warranted to fully understand the effects of IL-10 secreting B cells in atherosclerosis.

Other regulatory B cells

While typically regulatory B cells are associated with IL-10 producing B cells, the family of regulatory B cells has greatly expanded since the term was claimed[18]. It now also includes B cells expressing specific surface markers that can negatively regulate the immune response independent of IL-10 (Fig. 1).

For instance, Khan et al. identified a unique B cell subset that highly expresses programmed death ligand 1 (PD-L1)[34]. These PD-L1^{hi} B cells are potent regulators of T cell responses both *in vitro* and *in vivo*. In particular the expansion of follicular helper T cells (T_{FH}) that express the receptor for PD-L1 (PD-1) is negatively regulated by PD-L1^{hi} B cells[34]. T_{FH} cells are important modulators of the humoral immune response since they trigger and maintain the development of germinal centers. It has recently been demonstrated that this T_{FH}-germinal center axis develops during atherosclerosis, including isotype switching of antibodies from IgM to IgG[35]. Blockade or promotion of this axis results in attenuated or enhanced atherosclerosis in *apoE*$^{-/-}$ mice, respectively[35]. Since the PD-1/PD-L1 pathway has also shown its potential in modulating atherosclerosis, it will be very interesting to investigate the effects of the PD-L1^{hi} B cells in this context [36–38].

Another B cell subtype that has been shown to negatively regulate T cell responses is the so called killer B cell[39]. This type of B cell expresses Fas ligand (FasL, CD178) which is usually found on cytotoxic CD8$^+$ T cells and natural killer (NK) cells. Binding of FasL to its receptor Fas (CD95) leads to apoptosis in target cells. FasL$^+$ B cells are mainly found within the splenic CD5$^+$ population and can be induced by CD40 stimulation and IL-5[40]. Killer B cells are able to induce T_H cell apoptosis and promote tolerance[39,41], which are both crucial processes in treating atherosclerosis. In addition, soluble levels of FasL are clinically associated with a lower risk of cardiovascular disease[42,43]. Although it has not been directly shown that killer B cells secrete FasL, it remains of interest to study their influence on the pathophysiology of atherosclerosis.

A third type of B cell that has been reported to exert immune suppression depends on the ectoenzymes CD39 and CD73[44]. Both enzymes work towards the removal of extracellular ATP and increasing the level of extracellular adenosine (ADO), which is associated with atheroprotection[45]. CD39 is expressed on all B cells, but the expression of CD73 is restricted to a specific subset of B1 cells. Sorting of $CD73^-$ and $CD73^+$ B cells showed that only the latter were able to protect against experimental inflammatory colitis[44]. Ablation of CD73 results in increased neo-intima formation in a wire injury model[46] and increased atherosclerosis in *apoE*$^{-/-}$ mice[47]. Since these studies used total knockouts of CD73, the role of CD73 and/or CD39 on B cells in atherosclerosis has not yet been elucidated.

Innate response activator (IRA) B cells

In contrast to B_{regs}, additional B cell subsets have been identified that predominantly exert pro-inflammatory functions (Fig. 1). IRA B cells were identified as a unique B cell subset regarding their ontology, development and function. They are particularly characterized by the secretion of granulocyte macrophage colony stimulating factor (GM-CSF)[48]. GM-CSF is a pleiotropic cytokine that was first thought to be exclusively secreted by non-hematopoietic cells, macrophages and T cells. However, in response to LPS or sepsis a small population of peritoneal B1a cells is able to relocate to the spleen, secrete GM-CSF and hence protect against bacterial infection[48]. Several studies have been performed using total GM-CSF knockout mice on an apoE- or Ldlr-deficient background to examine the contribution of GM-CSF to atherosclerosis, but the results remain somewhat ambiguous. Gm-csf/apoE double knockout mice displayed an increase in atherosclerosis[49], while gm-csf/Ldlr double knockout mice showed no difference[50] or a sex-related reduction in plaque size (i.e. only in female mice)[51]. These conflicting results highlight the complex immune pathways of this cytokine and suggest that the context, timing and cellular source of GM-CSF might be important for the regulation of atherosclerosis.

Recently, it was shown by Hilgendorf et al. that during atherosclerosis progression, a small population of IRA B cells develops in the spleen[52]. Notably, they did not find any IRA B cells within the aorta, suggesting that B cells do not contribute to aortic GM-CSF and that they exert their effects indirectly. This was confirmed by showing that an increased expression of GM-CSF in the spleen during atherosclerosis progression resulted in an expansion of conventional $CD11c^+$ dendritic cells in the spleen. These in turn stimulated the proliferation of effector T cells and skewed them towards a Th1-dominant phenotype with a concomitant increase in antibodies

of the IgG2c isotype. This unfavorable immune response initiated by IRA B cells eventually culminated in an increase in atherosclerotic lesion size in *Ldlr*$^{-/-}$ mice[52]. Although this study elegantly showed that IRA B cells harbor atherogenic properties in the spleen of *Ldlr*$^{-/-}$ mice, it does not account for the differences found in the complete GM-CSF knock-out models between *Ldlr*$^{-/-}$ and *apoE*$^{-/-}$ mice. Since the increase in IRA B cells was most prominent in the *apoE*$^{-/-}$ mouse model, it would be expected that the contribution of B cells to the total levels of GM-CSF would be maximal in these mice. Along that line, *gm-csf*$^{-/-}$*apoE*$^{-/-}$ mice would most likely demonstrate a decrease in lesion size, however the opposite has been reported[49]. On the other hand, it could be suggested that since women are better protected against sepsis than men[53], they naturally contain more IRA B cells, which could at least partially explain the sex-difference found between females and males in *gm-csf*$^{-/-}$ *Ldlr*$^{-/-}$ mice[51].

Taken together, the study by Hilgendorf et al. was one of the first to report the effects of specific cytokine producing B cells on atherosclerosis, underscoring the immune-modulating effect B cells can have during atherosclerosis besides antibody production[52].

Concluding remarks

B cells can have a tremendous impact on atherosclerosis, however it is crucial to differentiate between the different B cell subsets regarding their effects on atherosclerosis. The increased knowledge on other B cell subtypes than B1/B2 and the additional roles besides antibody production in recent years, has also improved the understanding of B cells in cardiovascular disease. However, one drawback is that most subsets share surface markers, proving it difficult to pinpoint the individual effects to a specific subtype of B cells. In terms of therapeutic potential, the frequently used option of global B cell depletion with CD20 antibodies might not be the most favorable option. Ideally, treatments that either selectively induce protective B cells or limit the effects of atherogenic B cells could offer more potential.

In conclusion, there still is a lot to gain in exploring and harvesting the potential of B cells in the field of atherosclerosis.

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Bidirectional effects of IL-10^{+} regulatory B cells in *Ldlr*$^{-/-}$ mice

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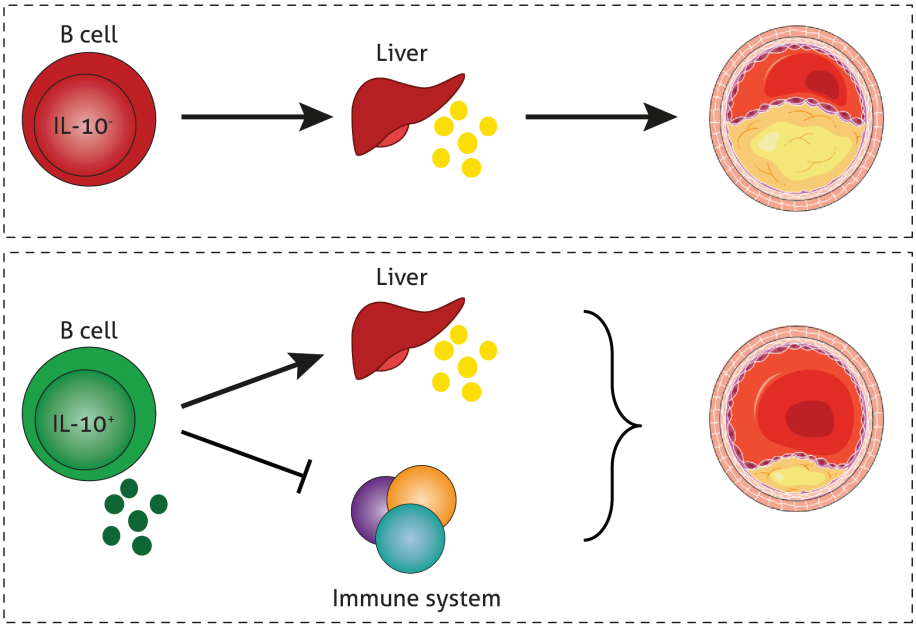
Abstract

Background and aims: Limiting the pro-inflammatory immune response is critical for the treatment of atherosclerosis. Regulatory B cells (Bregs) can modulate the immune response through interleukin-10 (IL-10). Current data regarding Bregs and atherosclerosis is scarce and conflicting.

Methods: In this study we investigated the frequency of IL-10$^+$ B cells during the development of atherosclerosis in low-density lipoprotein receptor-deficient (*Ldlr*$^{-/-}$) mice and studied the effect of adoptive transfer of IL-10$^+$ B cells on atherosclerosis.

Results: We found a very strong inverse correlation between atherosclerosis severity and the frequency of IL-10$^+$ B cells. This effect was cholesterol-independent and observed in spleen, draining lymph nodes and peritoneal cavity. To directly assess the effects of IL-10$^+$ B cells on atherosclerosis, we expanded IL-10$^+$ B cells *ex vivo* with anti-CD40 and selected pure and viable IL-10-secreting B cells and IL-10$^-$ B cells and adoptively transferred them to *Ldlr*$^{-/-}$ mice, respectively. Whereas IL-10$^-$ B cells were strongly atherogenic compared to control-treated mice, IL-10$^+$ B cells did not affect lesion size. Adoptive transfer of IL-10$^+$ B cells strongly reduced circulating leukocyte numbers and inflammatory monocytes. In addition, they decreased CD4$^+$ T cell activation and increased IL-10$^+$ CD4$^+$ T cell numbers. Interestingly, both IL-10$^+$ and IL-10$^-$ B cells exacerbated serum cholesterol levels and resulted in fatty livers, which potentially masked the beneficial effects of IL-10$^+$ B cells on atherosclerosis.

Conclusions: These findings underscore the strong immune-regulating function of IL-10$^+$ B cells and provide additional incentives to explore effective strategies that expand IL-10$^+$ B cells in atherosclerosis.



Graphical abstract.

Introduction

Cardiovascular disease remains a major global health problem and is mainly caused by atherosclerosis. Atherosclerosis is characterized by the build-up of lipids in the vascular wall which initiates a strong immune response, hence its classification as an autoimmune-like disorder[1]. At present, most treatment options are aimed at lipid lowering and do not address the ongoing immune response which perpetuates the disease. The recent success of the CANTOS trial strongly encourages research into novel therapeutic strategies that limit the immune reaction[2].

In the last decade, suppression of the immune reaction by regulatory B cells (Bregs) has received a lot of attention[3]. Bregs mediate the immune system primarily through interleukin-10 (IL-10) and it has been demonstrated that an imbalance in the number of Bregs strongly associates with multiple auto-immune disorders[4,5], including coronary artery disease[6]. In experimental models of auto-immune disorders, cellular therapy of Bregs have shown great potential, primarily via the induction of IL-10^{+} regulatory T cells[7–9]. Furthermore, it has been well-documented that IL-10 is protective in atherosclerosis[10]. However, there is a scarcity of research into Bregs and experimental atherosclerosis and the present data is conflicting[11–15]. While two groups report an atheroprotective effect of specific Breg subtypes[11,13], another study

using a mixed bone-marrow chimera model showed that B cell-derived IL-10 did not affect atherosclerosis[12]. These contradicting data clearly warrant more research into the contribution of Bregs to atherosclerosis.

A major hurdle in Breg research is the lack of a clearly defined immunophenotype. At present, there is a growing number of distinct Breg phenotypes defined by extracellular markers[7,16–21]. Most research up to date has focused on these Breg subset. However, IL-10$^+$ B cells are not limited to these subtypes and can be found within all B cell subsets, independent of their lineage or maturation and activation state[22]. Hence, IL-10 secretion is the primary mediator of immune suppression and remains the unique marker to classify Bregs. We thus aimed to determine in detail the role of IL-10$^+$ B cells in atherosclerosis by studying the frequency and status of IL-10$^+$ B cells during disease progression in low-density lipoprotein receptor-deficient mice (*Ldlr*$^{-/-}$) mice and by using an adoptive transfer model with viable IL-10-secreting B cells.

Material and methods

Animals

Male *Ldlr*$^{-/-}$ mice were bred in house and were kept under standard laboratory conditions. Mice were fed a normal chow diet or a Western-type diet (WTD) containing 0.25% cholesterol and 15% cocoa butter (Special Diet Services, Witham, Essex, UK). Diet and water were provided ad libitum. All injections were administered i.v. to the lateral tail vein in a total volume of 100 µl. During the experiments, mice were weighed, and blood samples were obtained by tail vein bleeding. At the end of experiments, mice were anaesthetized by a subcutaneous injection of a cocktail containing ketamine (40 mg/mL), atropine (50 µg/mL), and sedazine (6.25 mg/mL). Mice were exsanguinated by femoral artery transection followed by perfusion with PBS through the left cardiac ventricle. All animal work was approved by the Leiden University Animal Ethics Committee and the animal experiments were performed conform the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

Isolation of IL-10+ B cells

Viable IL-10-secreting B cells were isolated from *Ldlr*$^{-/-}$ donor mice using a regulatory B cell isolation kit according to protocol (Miltenyi Biotec). In short, B cells were isolated from the spleens of donor mice by negative selection. Secondly, these B cells were stimulated for 72h with 5 µg/ml of an agonistic CD40 antibody (Enzo

Life Science, clone FGK45). To stimulate active IL-10-secretion, B cells were further stimulated with LPS (50 µg/ml, *Escherichia coli* serotype O111:B4), PMA (50 ng/ml) and ionomycin (500 ng/ml) and labeled with an IL-10-catching antibody. Subsequently, the IL-10-secreting B cells were labelled with an IL-10 antibody conjugated to phycoerytrhin (PE) and finally isolated using magnetic PE-microbeads. The unlabeled cell fraction was also collected and determined as IL-10^{-} B cells. The isolated IL-10^{+} and IL-10^{-} B cells were consistently of a ≥90% purity.

Cell culture and cytokine determination

Isolated IL-10^{-} and IL-10^{+} B cells were cultured for 4 days with an agonistic CD40 antibody (5 µg/ml) after which IL-10 was measured in culture supernatants. For the T and B cell co-culture, naïve CD4^{+} T cells were isolated by magnetic selection according to the provided protocol (Miltenyi Biotec). 1x10^{5} CD4^{+} T cells were then plated in different ratios (1:2, 1:4, 1:8) with IL-10^{+} or IL-10^{-} B cells and stimulated with 5 µg/ml of plate-bound anti-CD3. After 72h of culture, cells were harvested and analyzed by flow cytometry. Concentrations of IL-10 in culture supernatants were measured using standard ELISA protocols (BD Bioscience).

Real-time quantitative PCR

RNA was extracted from isolated B cells by using Trizol reagent according to manufacturer's instructions (Invitrogen) after which cDNA was generated using RevertAid M-MuLV reverse transcriptase according to the instructions of the manufacturer (Thermo Scientific). Quantitative gene expression analysis was performed using Power SYBR Green Master Mix on a 7500 Fast Real-Time PCR system (Applied Biosystems). Gene expression of LIGHT (Forward: ATCTCACCAGGCCAACCCAGCA, Reverse: CCAGGCCAAGTCGTGTCTCCCATA) and lymphotoxin-alpha (Forward: CTGAAACCTGCTGCTCACCT, Reverse: ATGTCGGAGAAAGGCACGAT) was normalized to housekeeping genes (hydroxymethylbilane synthase, ribosomal protein 37 and glyceraldehyde 3-phosphate dehydrogenase).

Diet induced atherosclerosis

For the time-course experiment, age-matched (10-12 weeks old) *Ldlr*$^{-/-}$ mice were fed a WTD for 0, 3, 6 or 9 weeks (n=6/group). Mice were sacrificed at the same time-point after which the spleens were analyzed for the presence of IL-10^{+} B cells by flow cytometry. In a separate experiment, age-matched *Ldlr*$^{-/-}$ mice received a normal chow diet or WTD for 9 weeks (n=7/group). Multiple organs were harvested and processed for flow cytometry analysis. For the diet-switch experiment, *Ldlr*$^{-/-}$ mice were given either a WTD for 19 weeks, normal chow diet for 19 weeks or a WTD for 16 weeks and subsequently 3 weeks of normal chow diet (n=6/group).

These three weeks were determined by measuring the cholesterol levels during the switch to chow diet and the experiment was terminated when the cholesterol levels were normalized (data not shown). For the adoptive transfer experiment, at the start of WTD, 10-12 weeks old *Ldlr*$^{-/-}$ acceptor mice received 100 µl PBS or a cell suspension containing either 1x10^{6} IL-10^{-} or IL-10^{+} B cells (n=15/group). These injections were repeated at week 3 and 6 and mice were sacrificed after 9 weeks for analysis.

Flow cytometry

For the analysis of IL-10^{+} B cells, single cell suspensions were stimulated for 5 hours with LPS (50 µg/ml), PMA (50 ng/ml), ionomycin (500 ng/ml) and monensin (2 µM). For B10pro cells, B cells were first isolated with CD19-microbeads (Miltenyi Biotec) and subsequently stimulated for 48h with LPS (10 µg/ml) and/or an agonistic CD40 antibody (5 µg/ml). Secretion of cytokines by T cells was induced with stimulation of PMA, ionomycin and brefeldin A (5 µg/ml). For flow cytometry analysis, Fc receptors were blocked (2.4G2) and samples were stained with a dead/live marker (Fixable Viablity Dye) and anti-mouse fluorochrome-conjugated antibodies against B220 (RA3-6B2), CD19 (1D3), IL-10 (JES-16E3), CD4 (RM4-5), CD86 (GL1), CD69 (H1.2F3), CD25 (PC61.5), NK1.1 (PK136), CD11b (M1/70), Ly6-G (1A8), Ly-6C (HK1.4), CD62l (MEL-14), CD44 (IM7), IgM (II/41), IgD (11-26c), CD21 (7E9), CD23 (B3B4), CD24 (M1/69), TIM-1 (RMT1-4), CD1d (1B1), CD5 (53-7.3) or TNFα (MP6-XT22). All antibodies were purchased from Thermo Fisher Scientific or Becton Dickinson. FACS analysis was performed on a FACSCanto II (Becton Dickinson), and the acquired data were analyzed using FlowJo software. Gates were set according to unstimulated controls (only treated with monensin or brefeldin A) or to isotype and fluorescence minus one controls.

Serum measurements

During the experiment, blood was collected from the tail vein. After euthanasia, orbital blood was collected in EDTA-coated tubes. Whole blood cell counts were analyzed using the XT-2000iV hematology analyzer (Sysmex Europe GMBH, Norderstedt, Germany). Serum was acquired by centrifugation and stored at −20°C until further use. The total cholesterol levels in serum were determined using enzymatic colorimetric procedures (Roche/Hitachi, Mannheim, Germany). Precipath (Roche/Hitachi) was used as an internal standard. Lipoprotein profiles were determined by fractionation of 50 µL samples of serum using a Superose 6 column (3.2×300 mm, Smart-System; Pharmacia, Uppsala, Sweden) after which total cholesterol levels were determined in each fraction.

Liver lipid extraction and quantification

Triglycerides were extracted from liver tissue using Nonidet™ P 40 Substitute (Sigma-Aldrich Corp.). Liver tissue was homogenized with Nonidet™ P 40 Substitute, heated until 90°C and chilled on ice. This procedure was repeated once and subsequently insoluble material was removed by centrifugation. Cholesterol was extracted from liver tissue by the Folch extraction method[23]. Triglycerides and cholesterol concentration was determined by an enzymatic colorimetric assays.

Histology

To determine lesion size, cryosections (10 µm) of the aortic root were stained with Oil-Red-O and hematoxylin (Sigma-Aldrich). Sections with the largest lesion plus four flanking sections were analyzed for lesion size. Lipid content in liver sections was evaluated using the same Oil-Red-O staining. Collagen content in the lesion was assessed with a Masson's trichrome staining according to the manufacturers protocol (Sigma-Aldrich). Corresponding sections on separate slides were also stained for monocyte/macrophage content using a monoclonal rat IgG2b antibody (MOMA-2, 1:1000, AbD Serotec) followed by a goat anti-rat IgG-horseradish peroxidase antibody (1:100, Sigma-Aldrich) and color development.

Statistics

All data are expressed as mean ± SEM. Data were tested for significance using a Student's t-test for two normally distributed groups. Data from three groups or more were analyzed by an ordinary one-way ANOVA test followed by Tukey's post hoc test. When data from multiple time-points were tested, a repeated measures ANOVA was performed instead. Probability values of $p < 0.05$ were considered significant. All statistical analysis was performed using GraphPad Prism 7.0.

Results

IL-10^{+} B cells are significantly reduced during atherosclerosis development in *Ldlr*$^{-/-}$ mice

To examine the frequency of IL-10^{+} B cells during the development of atherosclerosis, we fed age-matched *Ldlr*$^{-/-}$ mice a Western type diet (WTD) for different time points (0, 3, 6 or 9 weeks) to induce various degrees of atherosclerosis. In general, two IL-10^{+} B cell types can be identified using flow cytometry; B10 and B10pro cells[24,25]. B10 cells readily secrete IL-10 after a short-term (5h) stimulation protocol (Fig. S1), while B10pro cells develop into IL-10-producing B cells after a long-term (48h) stimulation protocol with anti-CD40 and LPS. We found a gradual but sig-

nificant decrease of B10 cells in the spleen when mice were fed a WTD diet for longer periods (Fig. 1A). To assess whether the loss of IL-10$^+$ B cells correlated with disease severity, we measured the lesion size in aortic root sections and found a very significant inverse correlation between Bregs and atherosclerosis severity (Fig. 1B). Similarly, we observed an even stronger decrease in B10pro cells with increasing weeks of feeding the mice a WTD (Fig. 1C), which again highly correlated with increased atherosclerosis (Fig. 1D). In a separate experiment, we found that this effect was not spleen-specific but the loss of IL-10$^+$ B cells was also observed in the mediastinal lymph nodes draining from the aortic root and in the peritoneal cavity (Fig. 1E). In contrast, B cells in non-draining lymph nodes and in the circulation were

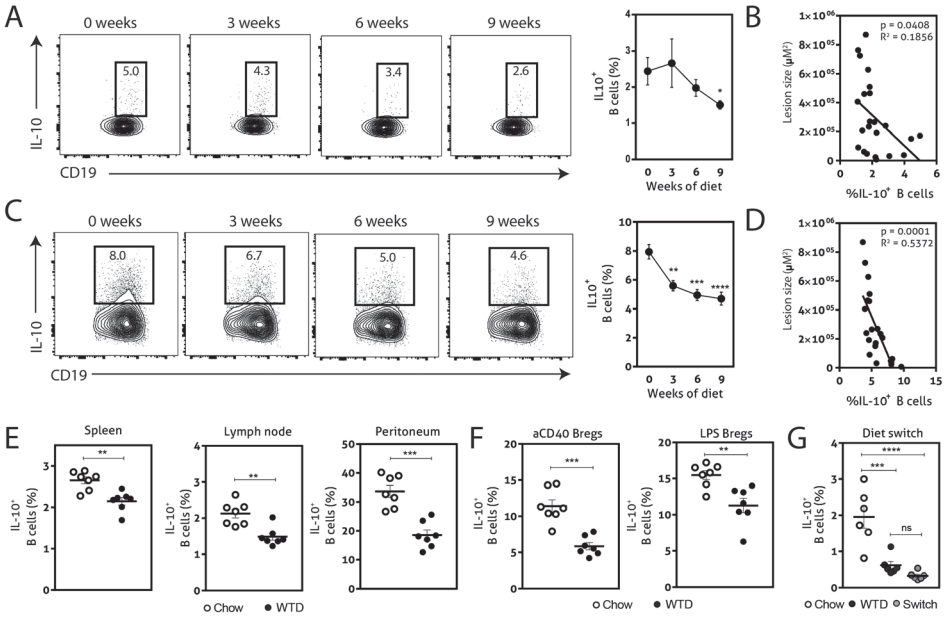


Figure 1. IL-10$^+$ B cells are significantly reduced during atherosclerosis development in *Ldlr*$^{-/-}$ mice. Age-matched *Ldlr*$^{-/-}$ mice were fed a Western type diet (WTD) or normal chow diet for varying periods and sacrificed at the same day. **(A)** IL-10$^+$ B cells in splenocytes were determined with flow cytometry after a short-term (5h) stimulation with LPS (50 µg/ml), PMA (50 ng/ml), ionomycin (500 ng/ml) and monensin (2 µM) (LPIM) and **(B)** correlated with lesion size in aortic root sections. **(C)** IL-10$^+$ B cells were determined after a long-term (48h) stimulation of isolated splenic B cells with LPS (50 µg/ml) and anti-CD40 (5 µg/ml) followed by LPIM stimulation and **(D)** correlated with lesion size in aortic root sections. **(E)** *Ldlr*$^{-/-}$ mice were fed a WTD or normal chow diet for 9 weeks after which IL-10$^+$ B cells were determined using LPIM stimulation in the spleen, lymph node and peritoneal cavity. **(F)** Identification of IL-10$^+$ B cells in mice fed a normal chow diet or a WTD for 9 weeks after stimulation of isolated splenic B cells with either LPS (50 µg/ml) or anti-CD40 (5 µg/ml) followed by LPIM stimulation. **(G)** *Ldlr*$^{-/-}$ mice were fed a normal chow diet for 19 weeks, a WTD for 19 weeks or a WTD for 16 weeks followed by a normal chow diet for 3 weeks (switch) to normalize cholesterol levels after which IL-10$^+$ B cells were determined in the spleen with LPIM stimulation. Data are shown as mean ± SEM (*p<0.05, ** p<0.01, ***p<0.001, ****p<.00001).

not affected by WTD-feeding (Fig. S2). We subsequently tested if a particular subset of regulatory B cells was affected during atherosclerosis development. This did not reveal a consistent reduction in specific subsets when we analyzed B cells in the spleen and lymph node (Fig. S3A-C). Along this line, we did not identify any differences in peritoneal subsets that pinpointed a particular B cell population that lost its IL-10 production after WTD diet feeding (Fig. S3D) We next wondered if the drastic reduction in B10pros was specifically a consequence of unresponsiveness to either LPS or anti-CD40. However, irrespective of the stimulus, flow cytometry revealed a highly significant decrease in the percentage of IL-10$^+$ B cells in the spleen of WTD-fed mice compared to chow mice (Fig. 1F). Since *Ldlr*$^{-/-}$ mice fed a WTD have increased levels of serum cholesterol, we investigated whether this was directly affecting the number of IL-10$^+$ B cells. Hence, we used a diet switch experiment by feeding *Ldlr*$^{-/-}$ mice either a normal chow diet, a WTD or a WTD followed by 3 weeks of a normal chow diet to normalize the cholesterol levels. We found that while the cholesterol levels in the diet-switched mice were fully normalized from 991 mg/dl to 240 mg/dl (data no shown), the loss of IL-10$^+$ B cells was not restored and remained significantly decreased compared to mice on a normal chow diet (Fig. 1G). Thus, IL-10$^+$ B cells showed a strong inverse correlation with atherosclerosis severity, but this was not directly correlated to cholesterol serum levels.

Adoptive transfer of IL-10$^+$ B cells leads to strong immune regulation

Given the strong decrease of IL-10$^+$ B cells during atherosclerosis progression, we wanted to explore strategies to restore the immune-regulating function of B cells. However, the low frequency and availability of IL-10$^+$ B cells severely limits the options of using them for cellular therapy. The practical difficulties can be overcome by either FACS-sorting Breg subpopulations based on extracellular markers[11,17] or to expand Bregs *ex vivo*, for example using anti-CD40 stimulation[8,26]. The drawback is that both of these options at best result in a B cell population that is an enriched mixture of IL-10$^+$ and IL-10$^-$ B cells. Since we found the greatest reduction in CD40-generated IL-10$^+$ B cells during atherosclerosis progression (Fig. 1F), we hypothesized that adoptive transfer of these cells would be most beneficial in the context of atherosclerosis. To directly assess the function of IL-10$^+$ B cells, we used a specialized kit to isolate viable IL-10-secreting B cells from anti-CD40 stimulated B cells that resulted in a 94.4% purity on average (Fig. S4). These IL-10$^+$ B cells displayed an IgM^{hi}IgD^{lo}CD21^{lo}CD24^{hi}CD1d^{hi}CD5$^+$ phenotype compared to IL-10$^-$ B cells (Fig. 2A), which highly corresponds with previously described IL-10$^+$ B cells from apolipoprotein E-deficient mice[24]. Furthermore, cytokine analysis revealed that while IL-10$^+$ B cells readily secrete IL-10 after several days of culture, we failed to detect IL-10 in supernatants of IL-10$^-$ B cells (Fig. 2B). Another major determinant of

regulatory B cells is their effect on CD4$^+$ T cell responses. In a co-culture experiment with CD4$^+$ T cells, our isolated IL-10$^+$ B cells significantly inhibited the development of TNFα$^+$ CD4 T cells. This inhibition was dependent on the B to T cell ratio and correlated with IL-10 levels in culture supernatants (Fig. 2C). Taken together, these data indicate that our culture protocol and isolation resulted in a highly pure population of IL-10$^+$ B cells with a clear regulatory phenotype and function. We adoptively transferred these IL-10$^+$ B cells to *Ldlr*$^{-/-}$ mice, which were subsequently fed a WTD for 9 weeks while PBS treatment and the negative fraction of the isolation (IL-10$^-$ B cells) were used as controls (Fig. S5).

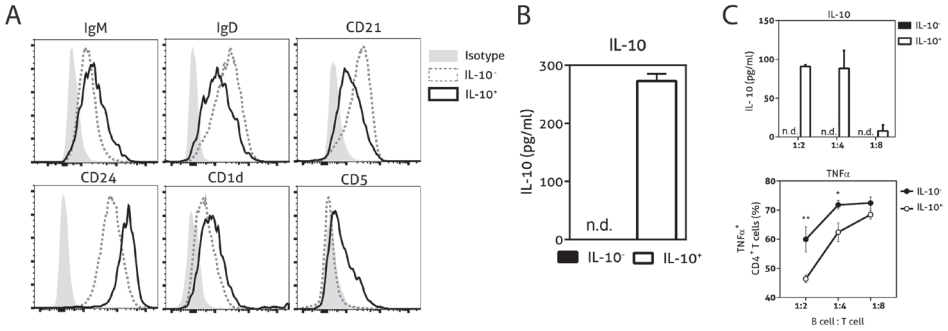


Figure 2. Characterization of IL-10$^+$ B cells. B cells were cultured with an agonistic CD40 antibody (5 μg/ml) for 72 hours after which they were stimulated with LPS (50 μg/ml), PMA (50 ng/ml) and ionomycin (500 ng/ml) to secrete IL-10. Subsequently IL-10$^-$ and IL-10$^+$ B cells were isolated. **(A)** Characterization of extracellular markers of IL-10$^-$ and IL-10$^+$ B cells with flow cytometry). **(B)** IL-10$^-$ and IL-10$^+$ B cells were cultured for 4 days with an agonistic CD40 antibody (5 μg/ml) after which IL-10 was measured in culture supernatants **(C)** Different ratios of IL-10$^-$ and IL-10$^+$ B cells were co-cultured with naïve CD4$^+$ T cells and stimulated with anti-CD3 (5 μg/ml). After 72 hours, IL-10 was measured in cultured supernatants with ELISA and TNFα$^+$ CD4 T cells were determined with flow cytometry.

Adoptive transfer of IL-10$^+$ B cells led to a very marked reduction in all circulating leukocytes compared to mice receiving PBS or IL-10$^-$ B cells (Fig. 3A). Monocytes contribute significantly to lesion formation and IL-10 is known to inhibit monocyte adhesion and affect the monocyte polarization[27,28]. Hence, we used flow cytometry to measure the number of inflammatory monocytes and found that mice treated with IL-10$^+$ B cells had a strong reduction in circulating inflammatory monocytes (Fig. 3B), indicating that adoptive transfer of IL-10$^+$ B cells led to a less atherosclerosis-prone phenotype of monocytes. Moreover, it is well-known that Bregs have strong effects on CD4$^+$ T cells[22]. Mice treated with IL-10$^+$ B cells showed a very strong increase in circulating IL-10$^+$ CD4$^+$ T cells compared to mice receiving PBS or IL-10$^-$ B cells (Fig. 3C). Similarly, we found that these circulating CD4$^+$ T cells showed a more naïve phenotype in mice treated with IL-10$^+$ B cells, while mice receiving IL-10$^-$ B cells demonstrated a

strong increase in effector memory CD4$^+$ T cells (Fig 3D). We also measured CD4$^+$ T cell activation in the lymph node draining from the aortic root and found a significant reduction in expression of CD69, CD25 and CD86 on CD4$^+$ T cells in mice treated with IL-10$^+$ B cells compared to mice receiving PBS or IL-10$^-$ B cells (Fig. 3E). This illustrated that the CD4$^+$ T cells are less activated in mice receiving IL-10$^+$ B cells compared to mice treated with PBS or IL-10$^-$ B cells. Taken together, these data clearly indicate that adoptive transfer of IL-10$^+$ B cell resulted in significant immune regulation.

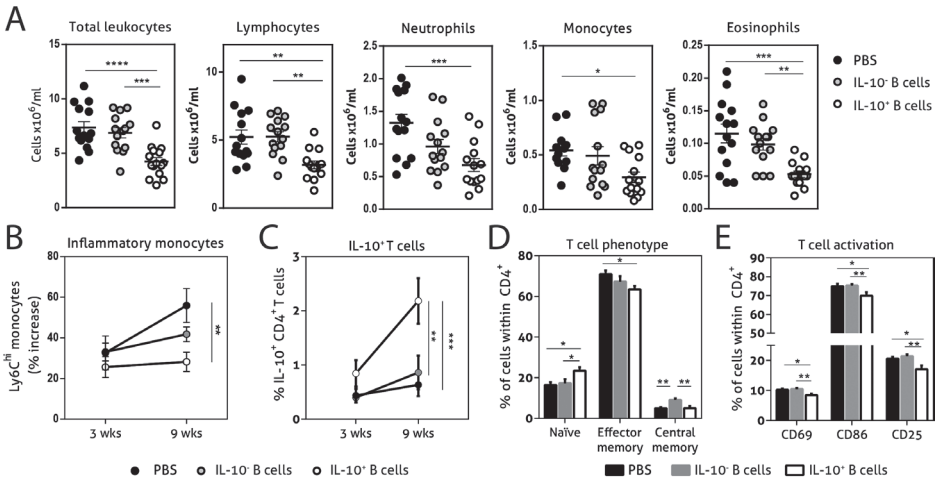


Figure 3. Adoptive transfer of IL-10$^+$ B cells leads to strong immune regulation. *Ldlr*$^{-/-}$ mice were put on a Western type diet for 9 weeks and received either three i.v. injections of PBS, IL10$^+$ or IL10$^-$ B cells (1x10^6 cells/injection). **(A)** Hematologic analysis of circulating white blood cells. Blood samples were analyzed with flow cytometry for (**B)** inflammatory monocytes (B220$^-$NK1.1$^-$ CD11b$^+$SSC^{lo}Ly-6G^{int}Ly-6C^{hi}), **(C)** IL-10$^+$CD4$^+$ T cells and **(D)** naïve (CD62l$^+$CD44$^-$), effector memory (CD62l$^-$CD44$^+$) or central memory (CD62l$^+$CD44$^+$) CD4$^+$ T cells. **(E)** Single cell suspensions of isolated lymph nodes were analyzed for activation markers on CD4$^+$ T cells with flow cytometry. Data are shown as mean ± SEM (*p<0.05, ** p<0.01, ***p<0.001, ****p<.00001).

IL-10$^-$ B cells are strongly atherogenic while IL-10$^+$ B cells do not affect lesion size

Considering the strong immune regulation after treatment with IL-10$^+$ B cells, we wondered what their effect on atherosclerosis development was. Hence, we measured lesion size and composition in the aortic root. We found that while treatment of mice with IL-10$^-$ B cells led to a drastic increase in atherosclerosis, treatment of mice with IL-10$^+$ B cells did not affect lesion size compared to PBS-treated mice (Fig. 4A). Similarly, lesion composition in terms of collagen and macrophage content did not differ between mice treated with PBS or IL-10$^+$ B cells. In contrast, lesions of mice that received IL-10$^-$ B cells showed a more advanced lesion phenotype with increased collagen and total macrophage area (Fig. 4B and 4C).

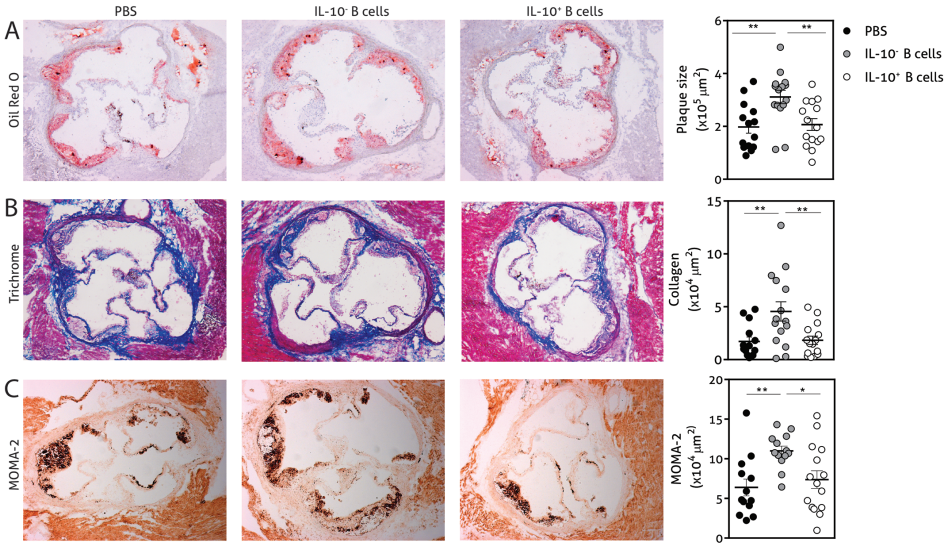


Figure 4. IL-10⁻ B cells are strongly atherogenic while IL-10⁺ B cells do not affect lesion size. *Ldlr*$^{-/-}$ mice were put on a Western type diet for 9 weeks and received either three i.v. injections of PBS, IL10^{+} or IL10^{-} B cells (1x10^{6} cells/injection). Representative pictures and quantification of aortic-root sections stained with **(A)** Oil Red O **(B)** Masson's trichrome staining and **(C)** MOMA-2. Data are shown as mean ± SEM (*p<0.05, ** p<0.01).

Adoptive transfer of both IL-10⁻ and IL-10⁺ B cells leads to exacerbated serum cholesterol levels

Given the strong evidence for an anti-atherogenic immune regulatory effect of IL-10^{+} B cells, we were surprised to find that adoptive transfer of IL-10^{+} B cells did not alter lesion size or composition. To explain this discrepancy, we explored other parameters, which could have masked the anti-atherogenic effects of IL-10^{+} B cells on the immune system. Considering the strong correlation of serum cholesterol levels with lesion size, we determined whether the cellular therapy had resulted in altered lipid levels. Indeed, we found differences in body weight (Fig. 5A) and an unexpected increase in total serum cholesterol levels in mice receiving IL-10^{+} as well as IL-10^{-} B cells (Fig. 5B), which was primarily caused by an increase in the levels of very-low density lipoprotein (Fig. 5C). We determined significant correlations of serum cholesterol levels and lesion size for mice treated with PBS or IL-10^{-} B cells, but this correlation was abolished in mice that received IL-10^{+} B cells (Fig. 5D). Since cholesterol is a key determinant for lesion development, this suggests that lipid-independent mechanisms interfered during the development of atherosclerosis. We further investigated the livers of mice treated with IL-10^{+} or IL-10^{-} B cells and found that these displayed increased Oil Red O staining compared to the PBS-treated mice (Fig. 5E). Moreover, we analyzed the lipid content of the livers and found a remark-

able increase in both cholesterol (Fig. 5F) and triglycerides (Fig. 5G) in mice that received B cell adoptive transfers compared to PBS-treated mice. Given the strong immune regulation in mice receiving IL-10^{+} B cells we believe that these effects have negated the atherogenic effects of increased cholesterol levels. In an effort to shed some light on the increased cholesterol levels, we measured the expression of lymphotoxin-alpha (LTα) and LIGHT on B cells after CD40-stimulation, since these molecules have previously been shown to regulate cholesterol levels in *Ldlr*$^{-/-}$ mice[29]. We found that CD40-stimulation of B cells significantly enhanced the expression of both LIGHT (Fig. S6A) and LTα (Fig. S6B). Our isolation protocol further required B cells to be stimulated with LPS, PMA and ionomycin and after this additional stimulus, expression of LIGHT and LTα remained high on B cells (Fig. S6A-B). Subsequently, we separated IL-10^{-} and IL-10^{+} B cells and measured the gene expression of LIGHT and LTα by qPCR and did not find any significant differences between IL-10^{-} and IL-10^{+} B cells (Fig. S6C). These findings might explain the increased cholesterol levels found in mice that received an adoptive transfer with IL-10^{-} or IL-10^{+} B cells.

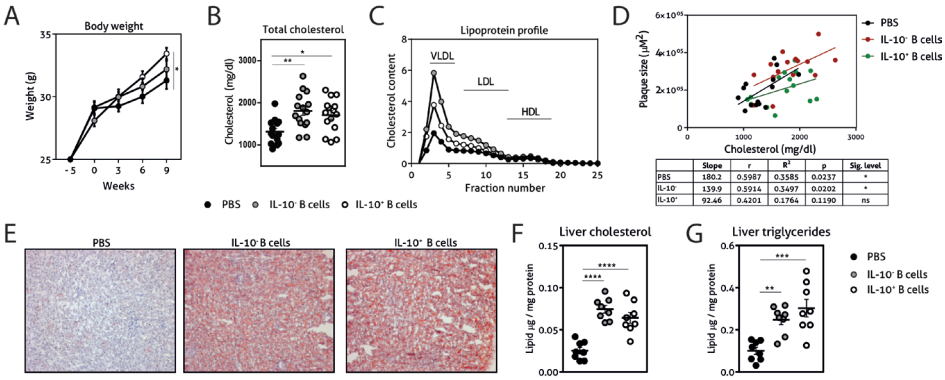


Figure 5. Adoptive transfer of both IL-10^{-} and IL-10^{+} B cells leads to exacerbated serum cholesterol levels. *Ldlr*$^{-/-}$ mice were put on a Western type diet for 9 weeks and received either three i.v. injections of PBS, IL10^{+} or IL10^{-} B cells (1x10^{6} cells/injection). (A) Body weight was assessed during the experiment. (B) Quantification of total serum cholesterol levels. (C) Characterization of the serum lipoprotein profile. (D) Correlations between serum cholesterol levels and plaque size in the aortic root. (E) Sections of liver tissue were stained with Oil-Red-O. (F) Quantification of extracted cholesterol content in the liver. (G) Quantification of extracted cholesterol content in the liver. Data are shown as mean ± SEM (*p<0.05, ** p<0.01, ***p<0.001, ****p<.00001).

Discussion

Since the role of Bregs in cardiovascular disease is currently under debate[11–13], we aimed to investigate the contribution of IL-10^{+} B cells to diet-induced atherosclerosis. IL-10^{+} B cells were drastically reduced during atherosclerosis development and

adoptive transfer of pure IL-10^{+} B cells limited the immune reaction. However, this was also associated with increased serum cholesterol levels and a fatty liver and this bidirectional effect prevented amelioration of atherosclerosis.

The increase in serum cholesterol levels after adoptive B cell transfer was a major unexpected finding of this study. There is a paucity of data examining the relation between immune cells and plasma cholesterol levels. While effects of dendritic cells[30] and T cells[29] have been described previously, a direct contribution of B cells to cholesterol homeostasis has not been recognized before. However, it has previously been shown that expression of LTα and LIGHT on CD4^{+} T cells is able to increase serum cholesterol levels[29]. Since it has also been shown that CD40-activation of B cells increases the expression of lymphotoxin on B cells[31,32], we measured the expression of LTα and LIGHT on B cells in our experiments. We found that our *ex vivo* stimulation protocol led to a considerable increase in extracellular expression of both proteins, which could potentially explain the exacerbated lipid levels found in this study. Despite the lack of a defined causal mechanism, these findings underscore that the immune response and plasma lipid levels are more interrelated in atherosclerosis than frequently thought. Additionally, the increased plasma lipids have undoubtedly influenced the atherosclerosis development in our study and could have masked the beneficial effects of IL-10^{+} B cells on atherosclerosis.

We did however find a strong atherogenic effect of IL-10^{-} B cells, which was not found using IL-10^{+} B cells. It is well-known that splenic B2 cells increase lesion size[33,34], in line with our findings using IL-10^{-} B cells, however we show here that pure splenic IL-10 producing B cells do not contain these atherogenic properties. Moreover, they limited the immune response and increased numbers of IL-10^{+} T cells. These findings corroborate with earlier data of an adoptive transfer study using CD21^{hi}CD23^{hi}CD24^{hi} B cells that both induced IL-10^{+} T cells and exerted vascular protection[11]. In contrast, another study investigating the role of B cell-derived IL-10 did not find any effect on atherosclerosis[12]. The authors used a mixed bone-marrow chimera model to establish a B cell-specific loss of IL-10, which did not influence lesion size. However, they also showed that IL-10 expression in the aorta was actually increased, possibly suggesting that IL-10 from other sources than B cells might compensate for the loss of B cell-derived IL-10[12]. Additional evidence for an atheroprotective role of Bregs was found by the observation that L-selectin deficiency decreased the aortic Breg cells and promoted atherosclerosis[13]. More recently, it was shown that induction of IL-10 producing B cells by a synergistic effect of BAFF and angiotensin II was associated with atheroprotection[33]. It has

also been shown that in human coronary artery disease Bregs have an impaired regulatory function[6].

Given the substantial reduction of IL-10^{+} B cells in atherosclerosis and the immune-regulating effects found in this study, we believe there is still ample evidence that IL-10^{+} B cells could protect against atherosclerosis. However, the lack of means to expand the number of Bregs *in vivo* still hampers this research. In this study we investigated a pure population of IL-10-secreting B cells, however the bidirectional effects induced by our culture protocol prevented an atheroprotective effect. In this respect, future research focusing on other strategies to induce Bregs in atherosclerosis remains of great interest.

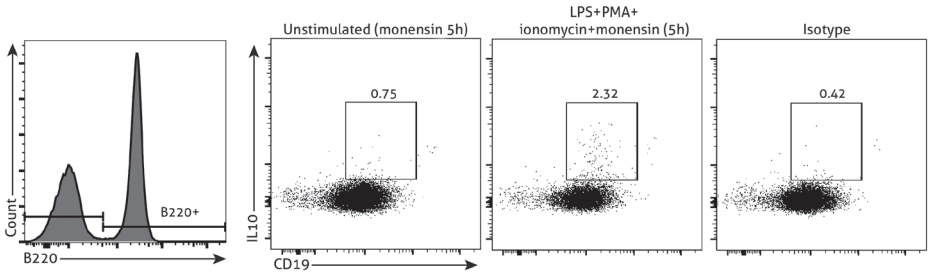
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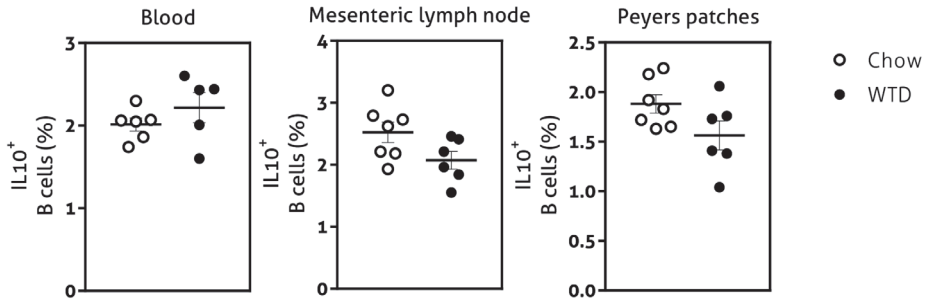
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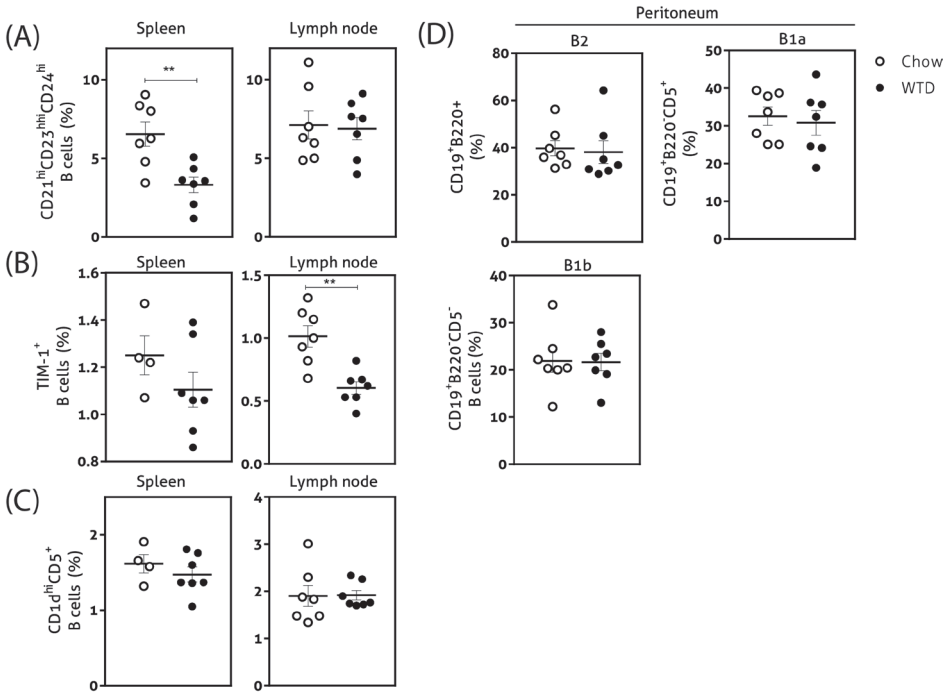
34. Kyaw T, Tay C, Hosseini H, Kanellakis P, Gadowski T, MacKay F, Tipping P, Bobik A, Toh B-H. Depletion of B2 but not B1a B cells in BAFF receptor-deficient ApoE mice attenuates atherosclerosis by potently ameliorating arterial inflammation. *PloS One* 2012;**7**: e29371.



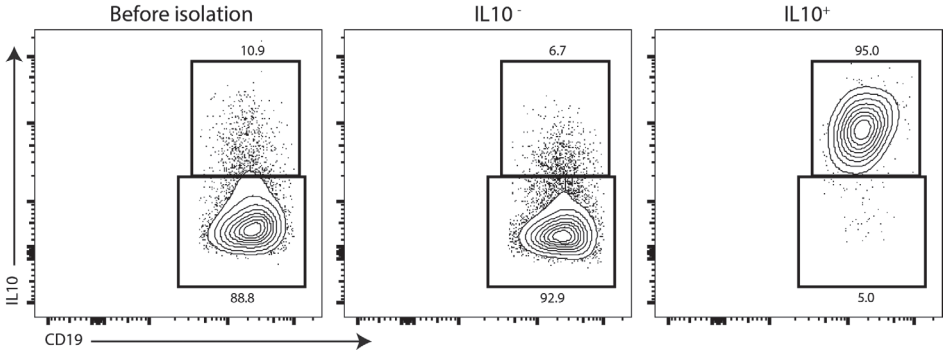
Supplementary Figure S1. Representative gating of IL-10^{+} B cells. Splenocytes were isolated and stimulated with a combination of LPS (50 µg/ml), PMA (50 ng/ml), ionomycin (500 ng/ml) and monensin (2 µM) for 5 hours. Fc receptors were blocked and samples were stained with anti-mouse fluorochrome conjugated antibodies against B220, CD19 and IL-10. After dead cells were excluded, B220^{+} cells were gated followed by gates for IL-10 and CD19 which were set according to unstimulated controls or to isotype controls.



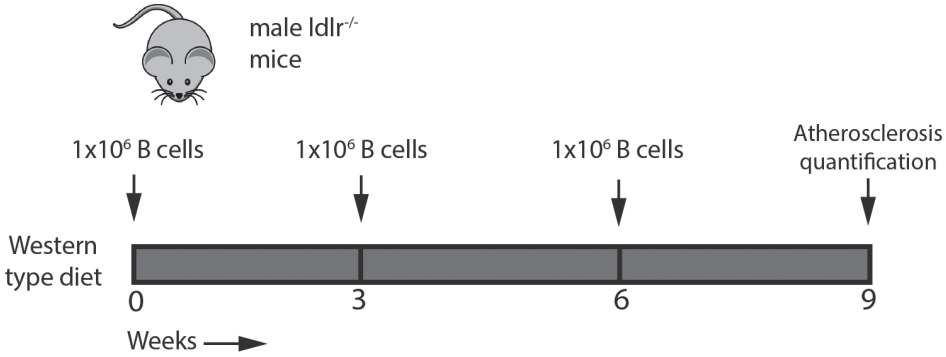
Supplementary Figure S2. IL-10^{+} B cells in the blood and non-draining lymph nodes are not affected by Western Type Diet (WTD). Mice were either put on WTD or normal chow diet for 9 weeks after which single cell suspensions of blood, mesenteric lymph nodes and Peyer's patches were stimulated with LPS+PMA+Ionomycin and Monensin for 5 hours. Fc receptors were blocked and samples were stained with anti-mouse fluorochrome conjugated antibodies against B220, CD19 and IL-10. Gates were set according to unstimulated controls or to isotype controls.



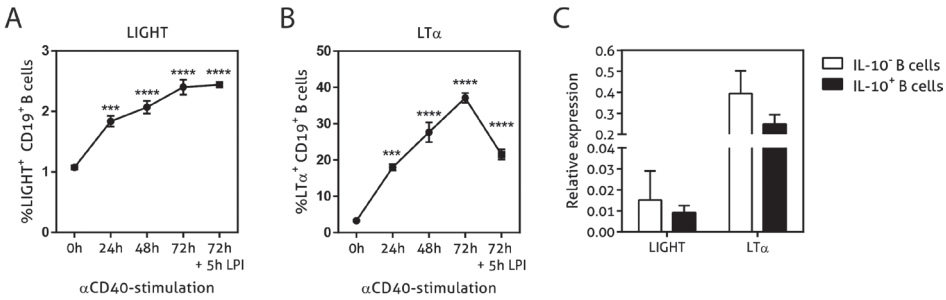
Supplementary Figure S3. Regulatory B cell subsets. *Ldlr*$^{-/-}$ mice were fed a WTD or normal chow diet for 9 weeks after which spleens and lymph nodes were analyzed with flow cytometry to determine **(A)** CD21^{hi}CD23^{hi}CD24^{hi} B cells, **(B)** TIM-1^{+} B cells and **(C)** CD1d^{hi}CD5^{+} B cells. **(D)** Single cell suspensions from the peritoneum were analyzed for B2 (CD19^{+}B220^{+}), B1a (CD19^{+}B220^{-}CD5^{+}) and B1b (CD19^{+}B220^{-}CD5^{-}) cells.



Supplementary Figure S4. Isolation of pure and viable IL-10^{+} B cells. B cells were cultured with an agonistic CD40 antibody (5 µg/ml) for 72 hours after which they were stimulated with LPS (50 µg/ml), PMA (50 ng/ml) and ionomycin (500 ng/ml) to secrete IL-10. Using a specialized IL-10-catching antibody, IL-10-secreting B cells were separated from non IL-10-secreting B cells using magnetic beads. Flow cytometry analysis of the isolated cells showed that this consistently resulted in a ≥95% purity of viable IL-10^{+} B cells.



Supplementary Figure S5. Experimental setup of adoptive transfer experiment. *Ldlr*$^{-/-}$ mice were put on a Western type diet for 9 weeks and received either three i.v. injections of PBS, IL-10$^+$ or IL-10$^-$ B cells (1x10^6 cells/injection) after which the mice were sacrificed and atherosclerosis was quantified.



Supplementary Figure S6. CD40-stimulation of B cells leads to increased lymphotoxin-alpha and LIGHT expression. Isolated CD19$^+$ B cells were stimulated for indicated periods of time with an agonistic CD40 antibody (5 µg/ml) with additional LPS, PMA and ionomycin stimulation where indicated. Subsequently, expression of (A) LIGHT and (B) lymphotoxin-alpha (LTα) were measured with flow cytometry. (C) IL-10$^-$ and IL-10$^+$ CD19$^+$ B cells were isolated after stimulation for 72 hours with an agonistic CD40 antibody followed by LPS, PMA and ionomycin stimulation for 5 hours. Subsequently, the expression of LIGHT and lymphotoxin-alpha (LTα) were measured with qPCR. Data are shown as mean ± SEM (***p<0.001, ****p<.00001).

TIM-1 mucin domain-mutant mice display exacerbated atherosclerosis

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Abstract

Increasing evidence has shown that immune checkpoint molecules of the T-cell immunoglobulin and mucin domain (TIM) family are associated with diverse physiologic and pathologic processes. Previous studies of the role of TIM-1 in atherosclerosis using anti-TIM-1 antibodies have yielded contradictory results. We thus aimed to investigate atherosclerosis development in TIM-1 deficient mice. In this study, mice with a specific loss of the TIM-1 mucin-domain (TIM-$1^{\Delta mucin}$) were used. Introduction of atherosclerosis in TIM-$1^{\Delta mucin}$ and C57BL/6 (WT) mice was performed with a single injection of a recombinant adeno-associated virus encoding murine PCSK9 (rAAV2/8-D377Y-mPCSK9). Subsequently mice were fed a Western type diet for 13 weeks and atherosclerosis development was quantified. TIM-$1^{\Delta mucin}$ mice developed significantly larger lesions in the aortic root and arch compared to WT mice. These lesions were composed of significantly more macrophages and showed a trend towards a larger necrotic core. The exacerbated atherosclerosis in TIM-$1^{\Delta mucin}$ mice was associated with a significant loss of IL-10^{+} B cells and regulatory B cell subsets compared to WT mice. TIM-$1^{\Delta mucin}$ mice also showed an increase in splenic and circulating leukocytes compared to WT mice, which was caused by an increase in B and $CD4^{+}$ T cells in the spleen, while no specific leukocyte population was changed in the circulation. Splenic $CD4^{+}$ T cell activation was not affected, but TIM-$1^{\Delta mucin}$ mice showed decreased $CD4^{+}$ T cell differentiation as measured by an increased number of naïve $CD4^{+}$ T cells. TIM-$1^{\Delta mucin}$ mice also displayed a dramatic reduction in Th2-associated immune response compared to controls but no changes in humoral immune response. In summary, TIM-$1^{\Delta mucin}$ mice displayed a profound impairment in IL-10^{+} B cells and an imbalance in the Th1/Th2 ratio, which associated with exacerbated atherosclerosis.

Introduction

Regulation of immune responses represents a promising option for the treatment of atherosclerosis[1]. Activation of the immune system is tightly controlled by immune checkpoint proteins and these molecules comprise an interesting group of therapeutic targets[2]. Immune checkpoint proteins are comprised of a diverse family of molecules, each with its own target cells and pathways. In general, immune checkpoint proteins are divided into stimulatory or inhibitory coreceptors, which are often associated with a proatherogenic and atheroprotective role, respectively[2]. Earlier data show that immune checkpoint molecules of the T-cell immunoglobulin and mucin domain (TIM) family are associated with a number of disorders including asthma, allergy and autoimmunity[3]. Eight murine members of the TIM gene family have been identified (TIM1-8), while in humans only three orthologues have been characterized (TIM1, -3, and -4). Structurally, TIM molecules are composed of an immunoglobulin V domain, a mucin-like domain, a transmembrane domain and a cytoplasmic tail[3]. There are distinct ligands for each TIM protein, however, there is some overlap between TIM-1 and TIM-4 since both molecules are able to bind phosphatidylserine which is expressed by apoptotic cells[4]. Additionally, it has been shown that TIM-4 is able to interact with TIM-1 as a ligand[5]. The cellular expression pattern of TIM proteins is quite broad, including T cells, B cells, macrophages and dendritic cells (DCs), highlighting the central role TIM molecules can play in the immune response.

We have previously explored the effects of TIM-1, TIM-3 and TIM-4 in atherosclerosis[6,7] and identified that antibody-mediated blockade of any of these TIM molecules results in exacerbated atherosclerosis, indicating a general protective role of TIM-signaling in atherosclerosis[6,7]. In contrast, others have recently shown that by using a different antibody clone, TIM-1 antagonism resulted in attenuated atherosclerosis[8]. TIM-1 is an immune checkpoint protein which is primarily expressed on $CD4^+$ helper type 2 cells[9], dendritic cells and regulatory B cells[10]. Principal studies suggested that TIM-1 is a stimulatory coreceptor since it induced T cell proliferation and cytokine production[9]. Later studies, however, showed that TIM-1 has a more complex immune checkpoint function[5,11]. It has been demonstrated that the administration of low doses of a soluble TIM-1 ligand (TIM-4) results in significant T cell inhibition, while higher doses increase T cell proliferation[5]. Additionally, it was shown that differential engagement by two TIM-1 antibodies could result in T cell expansion or inhibiton[11]. These data clearly demonstrate that TIM-1 signaling is highly dependent on ligand density and the extent of TIM-1 engagement[5,11]. This could potentially explain the differential effects of antagonistic TIM-1 antibodies on atherosclerosis[6,8].

Indeed, the anti-TIM-1 antibody used by Hosseini *et al.* (RMT1-10) has a low binding avidity and might be partial agonistic[11], while the antibody used in our previous experiments (3D10) is described as a non-activating antibody[12].

The contradictory results obscure the precise contribution of TIM-1 during atherosclerosis development and warrant additional research. To circumvent the complexities associated with antibody binding avidity and functionality, we aimed to examine the effects of TIM-1 in atherosclerosis using TIM-1-deficient mice. Earlier work showed that complete TIM-1 deficiency fails to show any marked phenotype[13,14]. However, others have previously shown that the mucin domain of TIM-1 has a crucial biological function in TIM-1 signalling[15]. Mice with a specific loss of the TIM-1 mucin-domain (TIM-1$^{\Delta mucin}$) retain the ligand binding IgV structure and express TIM-1 at normal levels. These mice do demonstrate a profound defect in regulatory B cells with associated development of systemic autoimmunity[15]. In this study, we thus examined the effect of defective TIM-1 signaling in TIM-1$^{\Delta mucin}$ mice on atherosclerosis.

Material and methods

Animals

Female C57BL/6 (WT) and TIM-1-mucin domain-deficient (TIM-1$^{\Delta mucin}$) mice[15] were bred in house and were kept under standard laboratory conditions. TIM-1$^{\Delta mucin}$ mice were provided by Dr. V. Kuchroo (Brigham and Women's Hospital). Mice were fed a Western-type diet (WTD) containing 0.25% cholesterol and 15% cocoa butter (Special Diet Services, Witham, Essex, UK). Diet and water were provided ad libitum. During the experiments, mice were weighed, and blood samples were obtained by tail vein bleeding. At the end of experiments, mice were anaesthetized by a subcutaneous injection of a cocktail containing ketamine (40 mg/mL), atropine (50 µg/mL), and sedazine (6.25 mg/mL). Mice were exsanguinated by femoral artery transection followed by perfusion with PBS through the left cardiac ventricle. All animal work was approved by the Leiden University Animal Ethics Committee and the animal experiments were performed conform the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

PCSK9-induced atherosclerosis

To induce atherosclerosis in WT and TIM-1$^{\Delta mucin}$ mice, 8-9 week old female mice were administered a single i.v. injection of rAAV2/8-D377Y-mPCSK9 ($5x10^{11}$ genome copies/mouse)[16]. This results in the rapid overexpression of proprotein convertase

subtilisin/kexin type 9 (PCSK9) and a significant decrease in hepatic low-density lipoprotein receptor (LDLR)[16]. For atherosclerosis development, mice were subsequently fed a WTD for 13 weeks before mice were sacrificed and relevant organs were harvested for analysis.

Flow cytometry

For the analysis of IL-10$^+$ B cells, single cell suspensions were stimulated for 5 hours with LPS (50 μg/ml), PMA (50 ng/ml), ionomycin (500 ng/ml) and monensin (2 μM). Secretion of cytokines by T cells was induced with stimulation of PMA, ionomycin and brefeldin A (5 μg/ml). For flow cytometry analysis, Fc receptors of single cell suspensions were blocked with an unconjugated antibody against CD16/CD32. Samples were then stained with a fixable viability marker (ThermoScientific) to select live cells and with anti-mouse fluorochrome-conjugated antibodies (see Supplementary Table 1). FACS analysis was performed on a FACSCanto II (Becton Dickinson) and the acquired data were analyzed using FlowJo software.

Proliferation assay

To measure T cell proliferation, 2x10^5 splenocytes were cultured in RPMI medium (supplemented with 10% FCS, L-glutamine and streptomycin/penicillin) for 72h. Cells were cultured with medium, oxidized low-density lipoprotein (oxLDL, 5 μg/ml) or stimulated with anti-CD3 (1.25 μg/ml) and anti-CD28 (1.25 μg/ml). Supernatant was harvested for cytokine analysis before splenocytes were pulsed with 3H-thymidine (0.5 μCi/well, Perkin Elmer, The Netherlands) for the last 16h. The amount of 3H-thymidine incorporation was measured using a liquid scintillation analyzer (Tri-Carb 2900R). Responses are expressed as the disintegrations per minute (DPM).

Cytokine determination

Cytokine levels in culture supernatants were measured using a Luminex bead-based multiplex assay (ProcartaPlex, Thermo Fisher Scientific) on a Luminex Instrument (MAGPIX). Recombinant cytokine standards (Thermo Fisher Scientific) were used to calculate cytokine concentrations and data were analyzed using Bio-Rad software.

Blood and serum analyses

During the experiment, blood was collected from the tail vein. After euthanasia, orbital blood was collected in EDTA-coated tubes. Whole blood cell counts were analyzed using the XT-2000iV hematology analyzer (Sysmex Europe GMBH, Norderstedt, Germany). Serum was acquired by centrifugation and stored at -20°C until further use. The total cholesterol levels in serum were determined using enzymatic colorimetric procedures (Roche/Hitachi, Mannheim, Germany). Precipath (Roche/

Hitachi) was used as an internal standard. Total serum titers of IgM, IgG1, IgG2c and oxidized LDL-specific antibodies were quantified by ELISA as previously described[17]. Serum levels of PCSK9 were measured by ELISA according to manufactures protocol (R&D systems).

Histology

To determine lesion size, cryosections (10 μm) of the aortic root and aortic arch were stained with Oil-Red-O and hematoxylin (Sigma-Aldrich). Sections with the largest lesion plus four flanking sections were analyzed for lesion size. Collagen content in the lesion was assessed with a Masson's trichrome staining according to the manufacturers protocol (Sigma-Aldrich). Corresponding sections on separate slides were also stained for monocyte/macrophage content using a monoclonal rat IgG2b antibody (MOMA-2, 1:1000, AbD Serotec) followed by a goat anti-rat IgG-horseradish peroxidase antibody (1:100, Sigma-Aldrich) and color development. All slides were analyzed with a Leica DM-RE microscope and LeicaQwin software (Leica Imaging Systems).

Statistics

All data are expressed as mean ± SEM. Data were tested for significance using a Student's t-test. Probability values of $p < 0.05$ were considered significant. All statistical analysis was performed using GraphPad Prism 7.0.

Results

TIM-1$^{\Delta mucin}$ mice display exacerbated atherosclerosis

Since we aimed to investigate the effects of impaired TIM-1 signaling in the context of atherosclerosis, we administered TIM-1$^{\Delta mucin}$ mice a single injection of a recombinant adeno-associated virus encoding for a gain-of-function for PCSK9 (rAAV2/8-D377Y-mPCSK9)[16]. This resulted in the rapid induction of circulating PCSK9 (Fig. S1A) and cholesterol (Fig. S1B) levels after mice were fed a WTD. Despite a significant lower body weight at the time of sacrifice (Fig. S1C), we found that TIM-1$^{\Delta mucin}$ mice showed a considerable increase in lesion development (Fig. 1A). We further characterized lesion composition and noticed that the macrophage content was significantly increased in lesions from TIM-1$^{\Delta mucin}$ mice compared to WT mice (Fig. 1B). Additionally, there were no differences in collagen content, but we found a trend towards a larger necrotic core area in lesions of TIM-1$^{\Delta mucin}$ mice compared to WT mice (Fig. 1C). We also analyzed lesion development in the aortic arch and found a dramatic increase in lesion size in TIM-1$^{\Delta mucin}$ mice compared to WT mice

(Fig. 1D). These data clearly indicate that TIM-1$^{\Delta mucin}$ mice developed exacerbated atherosclerosis.

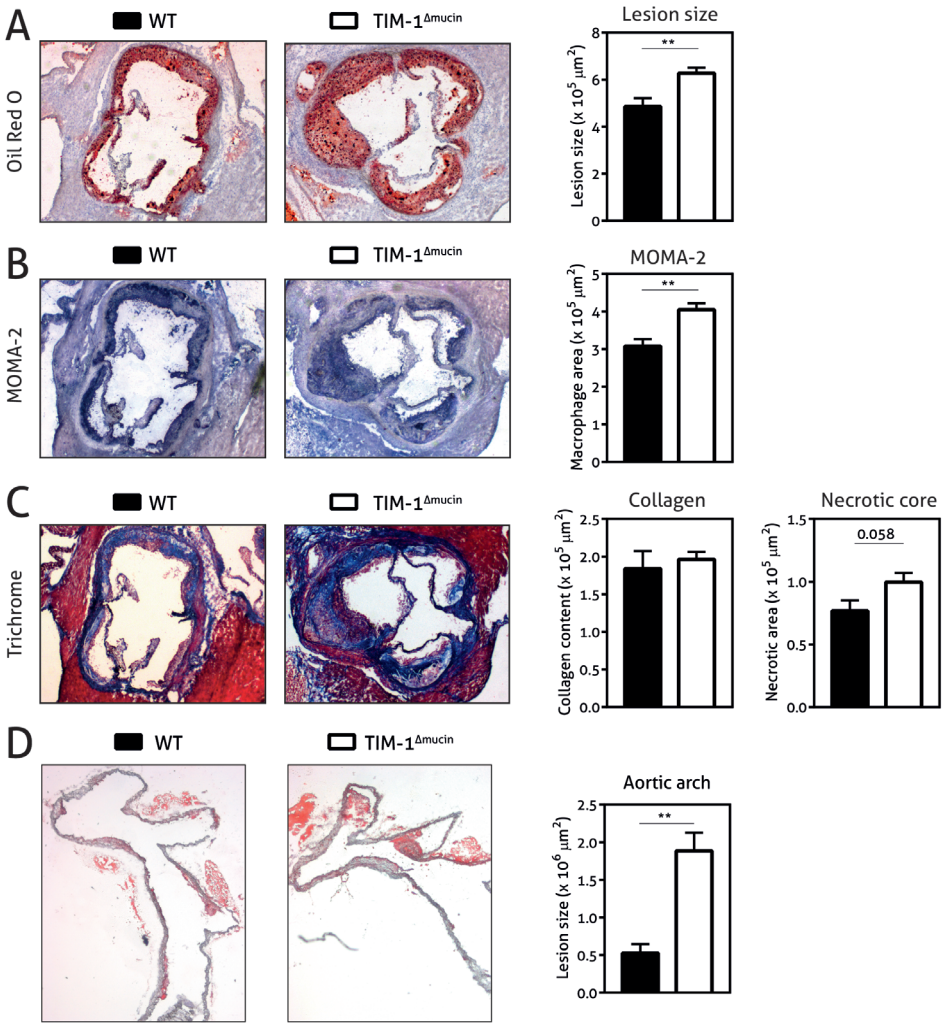


Figure 1. TIM-1$^{\Delta mucin}$ mice display exacerbated atherosclerosis. C57BL/6 (WT) and TIM-1-mucin domain-deficient (TIM-1$^{\Delta mucin}$) mice were administered a single i.v. injection of rAAV2/8-D377Y-mPCSK9 and subsequently fed a Western type diet for 13 weeks to induce atherosclerosis. After 13 weeks the aortic root was analyzed for **(A)** lesion size, which was determined with an Oil-Red-O and hematoxylin staining, **(B)** macrophage/monocyte content, which was determined with a MOMA-2 antibody and **(C)** collagen content and necrotic area, which were assessed using a Trichrome staining. **(D)** The aortic arch was analyzed for lesion size, which was determined with an Oil-Red-O and hematoxylin staining. Representative pictures are shown. Data are shown as mean ± SEM (**p<0.01), n=12-19/group for A-C and n=5-6/group for D.

TIM-1$^{\Delta mucin}$ mice show impaired IL-10 production by Bregs

In a previous study, it was demonstrated that the major impairment in TIM-1$^{\Delta mucin}$ mice is defective IL-10 production by B cells[15]. To assess if reduced IL-10-producing B cells (Bregs) could have contributed to the increased lesion development in our experiment, we measured circulating IL-10-producing B cells during the experiment. As shown in Figure 2A, IL-10^{+} B cells were reduced in TIM-1$^{\Delta mucin}$ mice compared to WT mice which reached significance at week 3 and 8. Additionally, the area under the curve showed that the total presence of circulating Bregs during the experiment was significantly reduced in TIM-1$^{\Delta mucin}$ mice compared to WT mice (Fig. 2B). Subsequently, we found a significant reduction of Breg cells in spleens of TIM-1$^{\Delta mucin}$ mice at sacrifice (Fig. 2C). Furthermore, we determined the level of well-known Breg subsets CD1d^{hi}CD5^{+} B cells[18] and CD9^{+} B cells[19,20] and found that these subsets were significantly lower in TIM-1$^{\Delta mucin}$ mice when compared to levels in WT mice (Fig. 2D and 2E). Since TIM-1 is also an important Breg marker[15,21], we measured the presence of TIM-1^{+} B cells, and found these cells were significantly decreased in TIM-1$^{\Delta mucin}$ mice compared to WT mice (Fig. 2F). These findings highlight that during atherosclerosis development, TIM-1$^{\Delta mucin}$ mice display an impaired IL-10 production by Bregs.

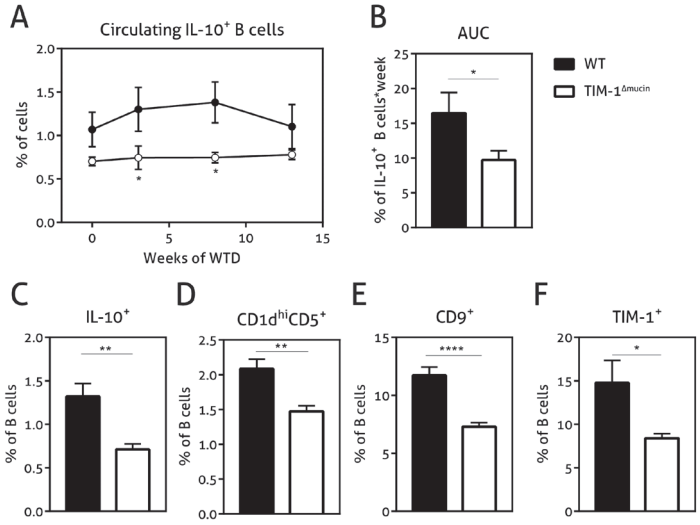


Figure 2. TIM-1$^{\Delta mucin}$ mice show impaired IL-10 production by Bregs. C57BL/6 (WT) and TIM-1-mucin domain-deficient (TIM-1$^{\Delta mucin}$) mice were administered a single i.v. injection of rAAV2/8-D377Y-mPCSK9 and subsequently fed a Western type diet for 13 weeks to induce atherosclerosis. **(A)** During the experiment, blood samples were obtained and single cell suspensions of blood leukocytes were stimulated for 5 hours with LPS, PMA, ionomycin and monensin after which the IL-10 positive B cells were determined with flow cytometry. **(B)** Quantification of the area-under-the curve of circulating IL-10^{+} B cells during the experiment. After 13 weeks, the number of **(C)** IL-10^{+} B cells, **(D)** CD1d^{hi}CD5^{+} B cells, **(E)** CD9^{+} B cells and **(F)** TIM-1^{+} B cells were determined in the spleen with flow cytometry. Data are shown as mean ± SEM (*p<0.05, **p<0.01, ****p<0.0001) and n=5-8/group.

Increased viable leukocytes in TIM-1$^{\Delta mucin}$ mice

Deficiencies in IL-10-producing B cells have previously been shown to induce an activated immune phenotype[15]. We examined if the development of atherosclerosis in TIM-1$^{\Delta mucin}$ mice led to a similar activation of the immune system. We first measured spleen weight (Fig. 3A) and the total number of spleen cells (Fig. 3B) and did not find differences between WT and TIM-1$^{\Delta mucin}$ mice. TIM-1 is involved in the recognition of apoptotic cells[22] and flow cytometry experiments displayed a consistent increase in the percentage of viable white blood cells both in the spleen and circulation of TIM-1$^{\Delta mucin}$ mice compared to WT mice (Fig. 3C). We further examined if specific leukocyte populations were increased, which revealed that the number of splenic CD4$^+$ T cells and CD19$^+$ B cells were significantly increased, while CD11b$^+$ leukocytes and dendritic cells were markedly decreased in the spleen of TIM-1$^{\Delta mucin}$ mice compared to WT mice (Fig. 3D). Analysis of circulating cells revealed a similar phenotype with increased CD19$^+$ B cells and a percentage of circulating monocytes (Fig. 3E).

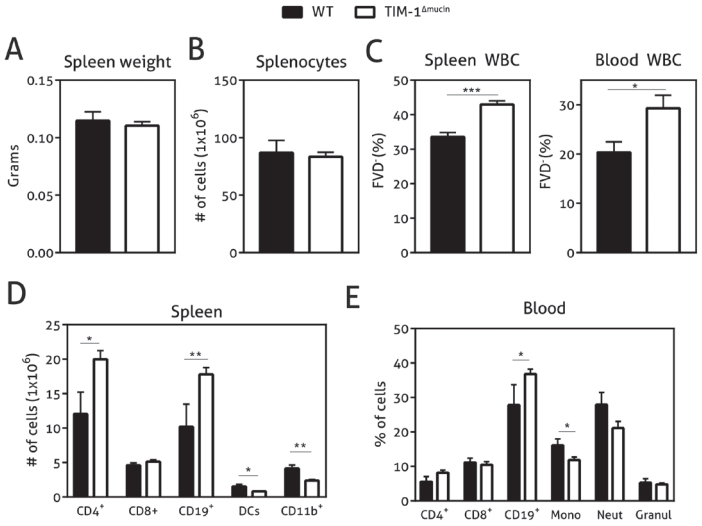


Figure 3. Increased viable leukocytes in TIM-1$^{\Delta mucin}$ mice. At sacrifice, (A) spleens of C57BL/6 (WT) and TIM-1-mucin domain-deficient (TIM-1$^{\Delta mucin}$) mice were weighed and (B) the total number of splenocytes were analyzed with a Sysmex hematolyzer. Using flow cytometry the percentage white bloods cells negative for Fixable Viability Dye (FVD) were assessed (C). Specific leukocyte populations in the (D) spleen and (E) circulation were determined. Data are shown as mean ± SEM (*p<0.05, **p<0.01, ***p<0.001) and n=5-8/group.

CD4$^+$ T cell differentiation but not activation is affected in TIM-1$^{\Delta mucin}$ mice

To investigate if the increase in splenic CD4$^+$ T cells was due to enhanced CD4$^+$ T cell activation, we first assessed the expression of CD69 and the proliferation marker Ki-67 in CD4$^+$ T cells. This, however, did not reveal any (statistical significant) differences between the different mice phenotypes (Fig. S2). Next, we isolated splenocytes and performed an *ex vivo* proliferation assay. Irrespective of the stimulus used (i.e. oxLDL or anti-CD3/anti-CD28), splenocytes from WT and TIM-1$^{\Delta mucin}$ mice showed a comparable proliferation (Fig. S3). Similarly, levels of IL-2 in the supernatants of anti-CD3/anti-CD28 stimulated splenocytes did not differ between

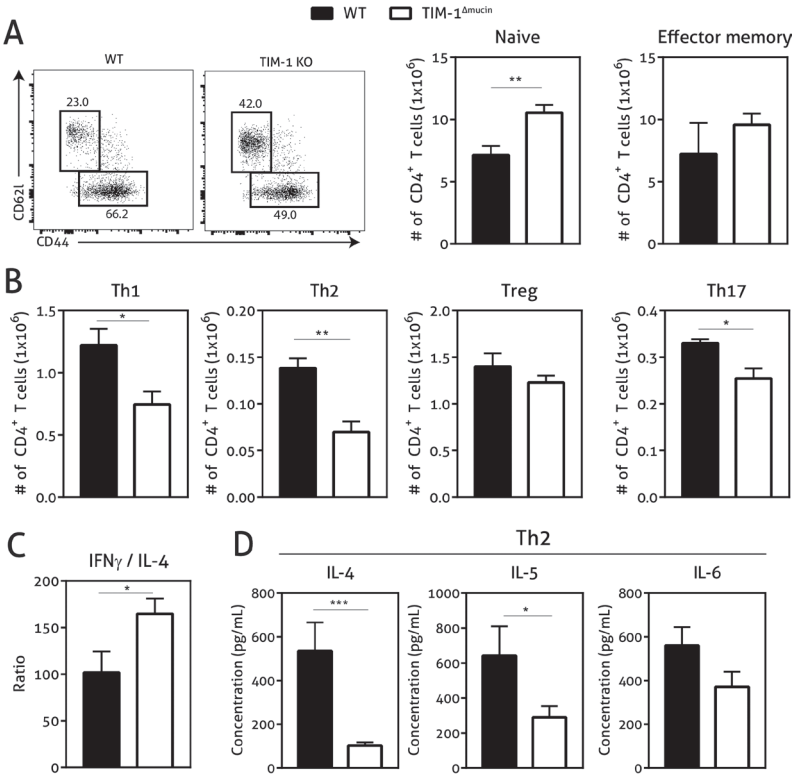


Figure 4. CD4$^+$ T cell response in TIM-1$^{\Delta mucin}$ mice. At sacrifice, splenocytes of C57BL/6 (WT) and TIM-1-mucin domain-deficient (TIM-1$^{\Delta mucin}$) mice were analyzed with flow cytometry to determine the number of **(A)** naïve (CD62l$^+$CD44^{hi}) and effector memory (CD62l$^-$CD44^{hi}) CD4$^+$ T cells and **(B)** Th1 (T-bet$^+$), Th2 (Gata-3$^+$), Treg (CD25$^+$FoxP3$^+$), Th17 (RORγt$^+$) CD4$^+$ T cells. **(C)** Single cell suspensions of splenocytes were stimulated for 5 hours with PMA, ionomycin and Brefeldin A after which intracellular IFNγ and IL-4 was measured and the ratio between IFNγ$^+$ and IL-4$^+$ CD4$^+$ T cells was determined. **(D)** Splenocytes were cultured in RPMI medium and stimulated with anti-CD3 (1.25 µg/ml) and anti-CD28 (1.25 µg/ml). After 72 hours, supernatant was harvested and analyzed with a multiplex assay for Th2-associated cytokines. Data are shown as mean ± SEM (*p<0.05, **p<0.01, ***p<0.001) and n=5-8/group.

WT and TIM-1$^{\Delta mucin}$ mice (Fig. S3). These data suggest that the activation of CD4$^+$ T cells in TIM-1$^{\Delta mucin}$ mice was not affected during atherosclerosis development. We subsequently tested whether the differentiation of CD4$^+$ T cells was altered, since TIM-1 appears to be primarily involved in Th2 responses[6,23]. We noticed that spleens of TIM-1$^{\Delta mucin}$ mice contained significantly more naïve CD4$^+$ T cells compared to spleens of WT mice (Fig. 4A), while no difference was found in the number of effector memory CD4$^+$ T cells. More detailed analysis of the CD4$^+$ T cell subsets showed that TIM-1$^{\Delta mucin}$ mice demonstrated a significant decrease in the number of Th1 and Th17 cells compared to WT mice, with an even more remarkable decrease in the number of Th2 cells (Fig. 4B). Subsequently, we used a five hour *ex vivo* stimulation with PMA and ionomycin to determine cytokine production by CD4$^+$ T cells from WT and TIM-1$^{\Delta mucin}$ mice. While we did not find any differences between TIM-1$^{\Delta mucin}$ and WT mice in IFNγ, IL-4 or IL-10 production with flow cytometry (Fig. S4), we noticed a significant shift in the IFNγ/IL-4 ratio indicating a reduced Th2-response (Fig. 4C). We wanted to further investigate this effect and measured additional cytokines in the culture supernatants of our *ex vivo* proliferation assay. In line with a reduced Th2-response, we did not observe any differences in Th1-, Th17- and Treg-associated cytokines in culture supernatants (Fig. S5), however we observed a large decrease in the Th2-associated cytokines IL-4 and IL-5 in culture supernatants from TIM-1$^{\Delta mucin}$ splenocytes compared to WT splenocytes (Fig. 4D). These data indicate that impaired TIM-1 signaling significantly decreased the differentiation of naïve CD4$^+$ T cells towards Th2 cells.

Humoral immunity is unaffected in TIM-1$^{\Delta mucin}$ mice

Isotype switching of B cells is strongly influenced by local cytokine production and given the increased number of splenic B cells and decrease in Th2 cells, we wondered if the humoral immunity was altered in TIM-1$^{\Delta mucin}$ mice. We measured total IgG1 (Fig. 5A) and IgG2c (Fig. 5B), which did not reveal any differences between the two groups. Given the shift we observed in the Th1/Th2 ratio, we examined the IgG2c/IgG1 ratio and found a slight but non-significant trend towards an increased ratio (Fig. 5C). Similarly, we did not find any differences in total oxLDL-specific IgG levels (Fig. 5D). It has recently been shown that treatment of mice with the RMT1-10 anti-TIM1 antibody resulted in increased peritoneal B1 cells and a concomitant increase in atheroprotective IgM antibodies[8]. In contrast, defective TIM-1 signaling did not influence peritoneal B1 cells numbers (Fig. 5E), total IgM levels (Fig. 5F) or oxLDL-specific IgM levels (Fig. 5G). Taken together, these data indicate that the humoral immunity was unaffected in TIM-1$^{\Delta mucin}$ mice.

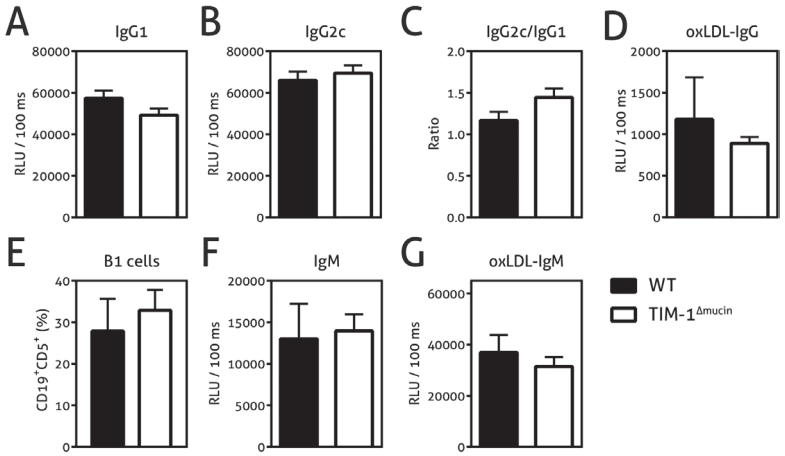


Figure 5. The humoral immunity is unaffected in TIM-1$^{\Delta mucin}$ mice. At sacrifice, serum from C57BL/6 (WT) and TIM-1-mucin domain-deficient (TIM-1$^{\Delta mucin}$) mice was analyzed for total **(A)** IgG1, **(B)** IgG2c and **(F)** IgM levels. Serum titers of **(D)** IgG and **(G)** specific for oxidized low-density lipoprotein (oxLDL) were measured. **(C)** Quantification of the IgG2c/IgG1 ratio. Flow cytometry was used to **(E)** quantify $CD19^+CD5^+$ B1 cells in the peritoneal cavity. Data are shown as mean ± SEM and n=5-8/group.

Discussion

TIM-1 has been associated with a broad number of inflammatory disorders. We and others have previously investigated the role of TIM-1 in atherosclerosis using anti-TIM-1 antibodies[6,8]. However, due to the complex nature of TIM-1 signaling these studies demonstrated contradicting results. It is clear that TIM-1 signaling is highly dependent on binding avidity, which varies with different anti-TIM-1 antibody clones. Here, we circumvented the antibody-associated difficulties by using TIM-1$^{\Delta mucin}$ mice and now show that TIM-1 has an overall protective effect during atherosclerosis development.

A previous study with TIM-1$^{\Delta mucin}$ mice revealed that up to 6 months of age these mice did not develop any overt auto-immune responses, but already showed a mild defect in B cell-derived IL-10 production[15]. With increasing age, or when crossbred onto an autoimmune genetic background, the Breg deficiency was exacerbated and resulted in concomitant increased numbers of B and T cells[15]. In this study, we show that the development of atherosclerosis in young TIM-1$^{\Delta mucin}$ mice (<6 months) was accompanied by profound impairment in B cell associated IL-10 production. Similarly, we observed leukocytosis in the spleen and circulation of TIM-1$^{\Delta mucin}$ mice, with primarily an increase in B cells and $CD4^+$ T cells. A major difference with the study of Xiao *et al.*, however, is our observation of increased naïve but not differ-

entiated $CD4^+$ T cells , while they observed increased $CD4^+$ T cell activation and differentiation TIM-$1^{\Delta mucin}$ mice[15]. They demonstrated that TIM-$1^{\Delta mucin}$ mice cross-bred with *Fas*-mutant lpr mice showed marked autoimmune responses, including enlarged lymphoid tissues, and hyperactive B and T cells. A potential reason for this contrast is that TIM-1 expression is highly dynamic and depending on the context and immune status its resulting effect can be either positive or negative coinhibition. *Fas*-deficient lpr mice on a C57BL/6 background already develop systemic autoimmunity[24], while atherosclerosis induces a much milder autoimmune response. This difference in immune status between the disease models could have influenced the $CD4^+$ T cell status in response to a lack in TIM-1 signaling. Additionally, we found that the number of splenic DCs was significantly reduced in our atherosclerotic TIM-$1^{\Delta mucin}$ mice. TIM-1 is constitutively expressed on DCs and TIM-1 signaling on DCs is associated with increased $CD4^+$ T cell effector responses[25]. Thus the reduced number and absence of TIM-1 on DCs could also have contributed to the decreased effector $CD4^+$ T cells in this study.

The two previous studies investigating the role of TIM-1 in atherosclerosis demonstrated contrasting results[6,8]. While we previously showed that antibody blockade of TIM-1 was associated with a shift in the Th1/Th2 balance and exacerbated atherosclerosis, Hosseini *et al.* demonstrated that TIM-1 inhibition resulted in atheroprotective IgM-producing B1a and IL-10^+ B cells[8]. In this study we investigated atherosclerosis in TIM-1 deficient mice and found a combination of these effects. It has been shown that TIM-1 is predominantly expressed on Th2 cells, while expression is low on Th1 cells[26]. In addition, TIM-1 is frequently associated with allergic diseases which are mainly Th2-driven[3]. In line with these findings, we observed a strong decrease in Th2 cells and Th2-associated cytokines. In contrast, Th1- and Th17-associated cytokines were similar in TIM-$1^{\Delta mucin}$ and WT mice, resulting in a significant switch in the Th1/Th2 balance. While our observation of increased naïve $CD4^+$ T cells would be expected to reduce atherosclerosis, atherosclerosis is primarily a Th1-driven disease and skewing towards a Th1-dependent immune response in TIM-$1^{\Delta mucin}$ mice will most likely have significantly contributed to the increased atherosclerosis development in these mice. Besides the effects on $CD4^+$ T cells, we also observed significant differences in IL-10^+ B cells in TIM-$1^{\Delta mucin}$ mice. It is well-known that TIM-1 has a prominent role in the maintenance and generation of IL-10^+ B cells[8,10,21]. In other auto-immune disorders, IL-10^+ B cells have shown great therapeutic potential, but their role in atherosclerosis is not clearly defined[27,28]. The work of Hosseini *et al*., however, showed that increased IL-10^+ and TIM-1^+ B cells after treatment with an partial agonistic TIM-1 antibody was associated with decreased atherosclerosis[8], indicating that the strong decrease in IL-10^+ B cells and Breg

subsets we found in TIM-1$^{\Delta mucin}$ mice could contribute to the exacerbated lesion development. Taken together, our work identified that mice completely deficient in effective TIM-1 signaling both showed an imbalance in the Th1/Th2 ratio as well as impaired numbers of IL-10^{+} B cells during atherosclerosis development.

There are also some interesting differences between our current study and earlier data. For instance, we previously reported that TIM-1 inhibition resulted in increased IgG1 antibodies[6] and others demonstrated that TIM-1 inhibition resulted in increased IgM production by B1a cells[8], while TIM-1$^{\Delta mucin}$ mice do not display any alterations in antibody levels. One explanation for these discrepancies might lie in the use of different experimental atherosclerosis models. While we previously used low-density lipoprotein receptor-deficient (*Ldlr*$^{-/-}$) mice on a WTD, Hosseini *et al.* investigated atherosclerosis development in apolipoprotein E-deficient mice. In the current study, we introduced atherosclerosis in TIM-1$^{\Delta mucin}$ mice by a PCSK9 adeno-associated virus yielding an *Ldlr*$^{-/-}$ phenotype. As alluded to above, the expression and function of TIM-1 is highly dynamic and therefore also susceptible for the underlying disease pathology. This is also illustrated by the fact that TIM-1 does not appear to have one unifying effect in inflammatory disorders[29]. Hence the subtle differences in experimental atherosclerosis models might have added to the observed differences found in these three studies.

In summary, this study has shed more light on the role of TIM-1 during atherosclerosis development. We show here that TIM-1Δmucin mice display a profound impairment of IL-10+ B cells and an imbalance in the Th1/Th2 ratio, which is associated with exacerbated lesion development. This work, combined with earlier data in mice, represents a firm foundation for future studies that could focus on elucidating the role of TIM-1 in human cardiovascular disease.

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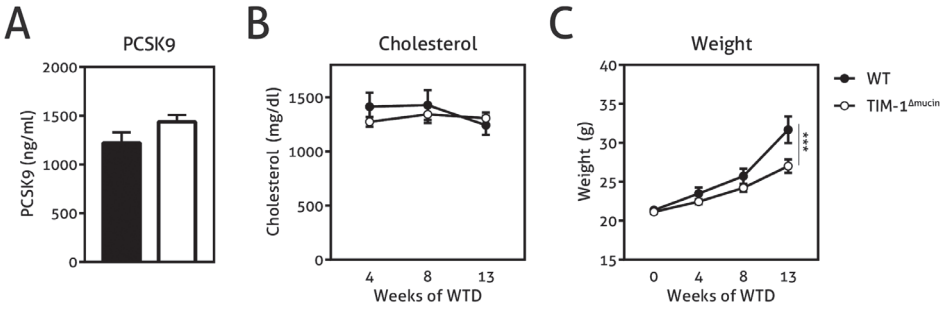


Figure S1. General characteristics of WT and TIM-1$^{\Delta mucin}$ mice. C57BL/6 (WT) and TIM-1-mucin domain-deficient (TIM-1$^{\Delta mucin}$) mice were administered a single i.v. injection of rAAV2/8-D377Y-mPCSK9 and subsequently fed a Western type diet for 13 weeks to induce atherosclerosis. **(A)** Circulating levels of proprotein convertase subtilisin/kexin type 9 (PCSK9) were measured after 13 weeks. **(B)** Serum cholesterol levels and **(C)** weight were monitored during the experiment. Data are shown as mean ± SEM (***p<0.001).

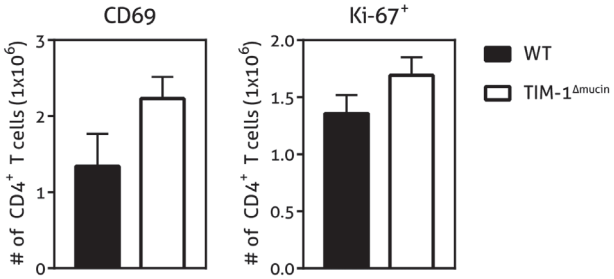


Figure S2. CD4$^+$ T cell activation is not affected in TIM-1$^{\Delta mucin}$ mice. C57BL/6 (WT) and TIM-1-mucin domain-deficient (TIM-1$^{\Delta mucin}$) mice were administered a single i.v. injection of rAAV2/8-D377Y-mPCSK9 and subsequently fed a Western type diet for 13 weeks to induce atherosclerosis. After 13 weeks single cells suspensions of the spleen were analyzed for CD69$^+$ and Ki-67$^+$ CD4$^+$ T cells. Data are shown as mean ± SEM.

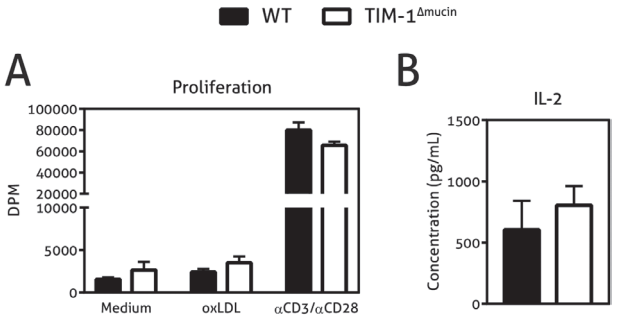


Figure S3. No differences in T cell proliferation in TIM-1$^{\Delta mucin}$ mice. C57BL/6 (WT) and TIM-1-mucin domain-deficient (TIM-1$^{\Delta mucin}$) mice were administered a single i.v. injection of rAAV2/8-D377Y-mPCSK9 and subsequently fed a Western type diet for 13 weeks to induce atherosclerosis. **(A)** After 13 weeks, 2x10^5 splenocytes were cultured in RPMI medium and stimulated with oxidized low-density lipoprotein (oxLDL; 5 µg/ml) or anti-CD3 (1.25 µg/ml) and anti-CD28 (1.25 µg/ml). Cells were pulsed with 3H-thymidine (0.5 µCi/well) for the last 16h. The amount of 3H-thymidine incorporation was measured and expressed as the disintegrations per minute (DPM). **(B)** Levels of IL-2 in culture supernatants of anti-CD3 and anti-CD28. Data are shown as mean ± SEM.

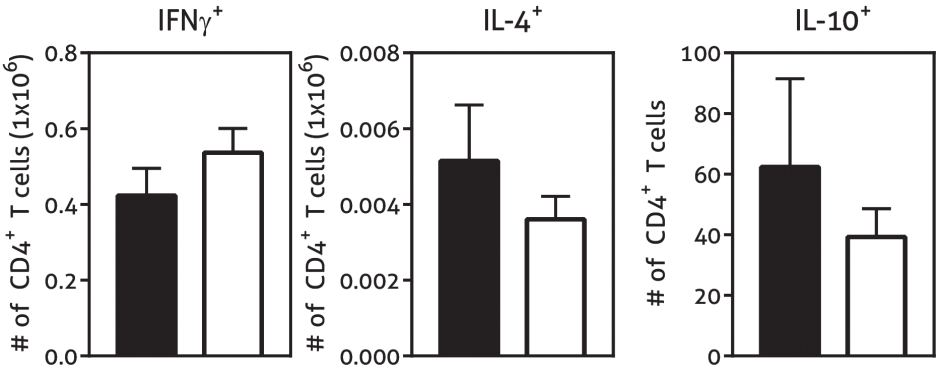


Figure S4. No differences in intracellular cytokines of CD4$^+$ T cells in TIM-1$^{\Delta mucin}$ mice. C57BL/6 (WT) and TIM-1-mucin domain-deficient (TIM-1$^{\Delta mucin}$) mice were administered a single i.v. injection of rAAV2/8-D377Y-mPCSK9 and subsequently fed a Western type diet for 13 weeks to induce atherosclerosis. After 13 weeks, single cell suspensions of splenocytes were stimulated for 5 hours with PMA, ionomycin and Brefeldin A after which intracellular IFNγ, IL-4 and IL-10 was determined within the CD4$^+$ population. Data are shown as mean ± SEM.

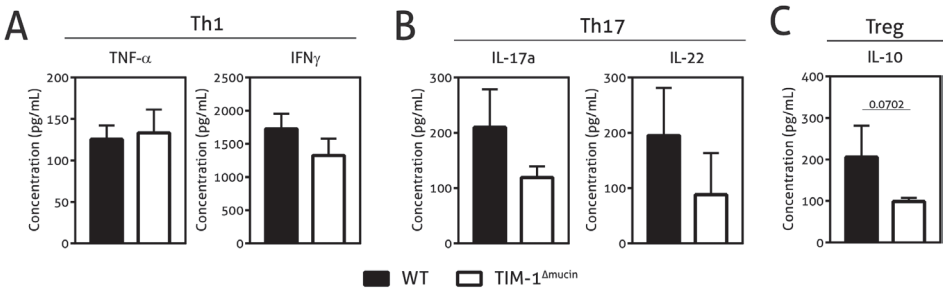


Figure S5. Cytokine production of CD4$^+$ T cells in TIM-1$^{\Delta mucin}$ mice. C57BL/6 (WT) and TIM-1-mucin domain-deficient (TIM-1$^{\Delta mucin}$) mice were administered a single i.v. injection of rAAV2/8-D377Y-mPCSK9 and subsequently fed a Western type diet for 13 weeks to induce atherosclerosis. After 13 weeks, 2x10^5 splenocytes were cultured in RPMI medium and stimulated with anti-CD3 (1.25 µg/ml) and anti-CD28 (1.25 µg/ml). After 72 hours, supernatant was harvested and analyzed with a multiplex assay for **(A)** Th-1 associated cytokines, **(B)** Th17-associated cytoines and **(C)** IL-10$^+$. Data are shown as mean ± SEM.

IFNγ-stimulated B cells inhibit T follicular helper cells and protect against atherosclerosis

Submitted

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Abstract

B and T cells are interconnected in the T follicular helper – germinal center B cell (T_{FH}-GC B cell) axis, which is hyperactive during atherosclerosis development and loss of control along this axis results in exacerbated atherosclerosis. Inhibition of the T_{FH} – GC B cell axis can be achieved by providing negative co-stimulation to T_{FH} cells through the PD-1/PD-L1 pathway. Therefore, we investigated a novel therapeutic strategy using PD-L1-expressing B cells to inhibit atherosclerosis. We found that IFNγ-stimulated B cells significantly enhanced PD-L1 expression and limited T_{FH} cell development. To determine whether IFNγ-B cells can reduce collar-induced atherosclerosis, a*poE*$^{-/-}$ mice fed a Western-type diet were treated with PBS, B cells or IFNγ-B cells for a total of 5 weeks following collar placement. IFNγ-B cells significantly increased PD-L1^{hi} GC B cells and reduced plasmablasts. Interestingly, IFNγ-B cells–treated mice show increased atheroprotective Tregs and T cell-derived IL-10. In line with these findings, we observed a significant reduction in total lesion volume in carotid arteries of IFNγ-B cells-treated mice compared to PBS-treated mice and a similar trend was observed compared to B cell-treated mice. In conclusion, our data show that IFNγ-stimulated B cells strongly upregulate PD-L1, inhibit T_{FH} cell responses and protect against atherosclerosis.

Introduction

Cardiovascular disease (CVD) remains a major global health problem despite great developments in diagnosis and treatment. The underlying cause for CVD is atherosclerosis, which is a chronic autoimmune-like disease and is characterized by the formation of lipid-rich lesions in the arteries. The current available treatments are aimed at lipid lowering and lead to a 25-30% relative risk reduction[1,2], indicative of an urgent need for novel disease-modifying drugs. In the last decade, accumulating evidence identified the immune system as a major contributor to the pathology of atherosclerosis[3]. For this reason, considerable effort has been devoted to restore the dysregulation of the immune system and inflammatory pathways in atherosclerosis.

B and T-lymphocyte dependent immune responses play a key role in the pathophysiology of atherosclerosis and the role of most B and T cell subsets in atherosclerosis is now well-defined. For instance, B1 cells have shown a consistent atheroprotective effect[4–7], while in multiple studies B2 cells were seen to contribute to atherosclerosis[8–11]. In addition, the involvement of Th1, Th2 and Treg cells in atherosclerosis has also been examined comprehensively (reviewed in[3]). In contrast, only recently the contribution of other leukocyte subpopulations such as follicular (FO) B cells, marginal zone (MZ) B cells and follicular helper T cells (T_{FH}) to atherosclerosis have been identified. Although FO B cells, MZ B cells and T_{FH} cells are radically different cell types, they appear to be interconnected in the T_{FH} – Germinal center B cell axis. FO B cells enter germinal centers, subsequently undergo proliferation and isotype switching and can differentiate in short-lived plasmablasts, which can further differentiate into long-lived plasma cells or memory cells. FO B cells promote the recruitment of T_{FH} cells and the generation of germinal centers through expression of inducible co-stimulator ligand[12]. Previously, it has been shown that the T_{FH} – GC B cell axis is hyperactivated and promotes lesion formation in both apolipoprotein E-deficient (*apoE*$^{-/-}$) mice[13] and low-density lipoprotein receptor-deficient mice fed a hypercholesterolemia-inducing diet[14]. In addition, both T_{FH} cells and FO B cells are proatherogenic and can aggravate atherosclerosis[15 16].

The T_{FH} – GC B cell axis can be regulated by the co-inhibitory programmed death-1 (PD-1)/PD-L1 pathway. T_{FH} cells highly express PD-1 and their accumulation can be controlled by MZ B cells which express PD-L1. In response to high cholesterol levels, MZ B cells upregulate the expression of PD-L1 and thereby regulate T_{FH} cell accumulation which limits an exacerbated adaptive immune response.[14] However, this mechanism fails to completely arrest disease development. Interestingly, in autoimmune encephalomyelitis, it has been shown that adoptive transfer of B cells

expressing high levels of PD-L1 limited disease severity[17]. Whether PD-L1 expressing B cells can also be used therapeutically to inhibit atherosclerosis development has not yet been reported. In this study, we therefore induced PD-L1-expressing B cells and investigated whether adoptive transfer of these cells could inhibit atherosclerosis development.

Methods

Animals

All animal work was approved by the Leiden University Animal Ethics Committee and the animal experiments were performed conform the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. Female C57BL/6, B6.SJL-PtprcaPepcb/BoyCrl (also known as CD45.1) and apolipoprotein E-deficient (*apoE*$^{-/-}$) mice were bred in house and kept under standard laboratory conditions. Diet and water were provided ad libitum. All injections were administered i.v. to the lateral tail vein in a total volume of 100 µl. During the experiments, mice were weighed, and blood samples were obtained by tail vein bleeding. At the end of experiments, mice were anaesthetized by a subcutaneous injection of a cocktail containing ketamine (40 mg/mL), atropine (50 µg/mL), and sedazine (6.25 mg/mL). Mice were bled and perfused with phosphate-buffered saline (PBS) through the left cardiac ventricle.

Cell culture

B cells were isolated from splenocytes of C57BL/6 or *apoE*$^{-/-}$ mice using CD19^{+} microbeads (Miltenyi Biotec) and cultured in complete RPMI medium. Isolated B cells were cultured with different concentrations of heat killed *Staphylococcus aureus* (Invivogen), B-cell activating factor (BAFF; R&D systems) or interferon-gamma (IFNγ; ThermoFisher) for 24 hours. For co-culture experiments, CD4^{+} T cells were isolated from splenocytes of C57BL/6 mice using a CD4^{+} T cell isolation kit (Miltenyi Biotec). For some experiments, the CD4^{+} T cells were labeled using a CellTrace Violet Cell Proliferation kit (ThermoFisher) according to the instructions of the manufacturer. B and T cells were co-cultured and stimulated with an agonistic plate-bound CD3 antibody (5 µg/ml) for 72 hours. For adoptive transfer experiments, B cells were isolated from splenocytes of *apoE*$^{-/-}$ or CD45.1 mice, cultured in RPMI medium and stimulated for 24 hours with 20 ng/ml of IFNγ. Subsequently, cells were washed, checked for purity using flow cytometry and resuspended for injections with PBS. For the injection of untouched B cells, B cells were freshly isolated from splenocytes of *apoE*$^{-/-}$ or CD45.1 mice and directly used for adoptive transfer experiments.

Real-time quantitative PCR

RNA was extracted from cultured B cells by using Trizol reagent according to manufacturer's instructions (Invitrogen) after which cDNA was generated using RevertAid M-MuLV reverse transcriptase according to the instructions of the manufacturer (Thermo Scientific). Quantitative gene expression analysis was performed using Power SYBR Green Master Mix on a 7500 Fast Real-Time PCR system (Applied Biosystems). Gene expression was normalized to housekeeping genes. Primer sequences are available in Supplementary table 1.

In vivo experiments

For all *in vivo* experiments, *apoE*$^{-/-}$ mice were used. *ApoE*$^{-/-}$ mice were fed a Western-type diet containing 0.25% cholesterol and 15% cacao butter (SDS, Sussex, UK). For the pilot study, mice were pre-fed a Western-type diet for 2 weeks and were subsequently treated with 2x10^6 freshly isolated or cultured B cells and sacrificed after three days. For atherosclerosis experiments, carotid artery plaque formation was induced after two weeks of Western-type diet feeding by perivascular collar placement in these mice as described previously[35]. Mice continued to be fed a Western-type diet for 5 weeks and during this period received 3 injections with either freshly isolated B cells (2x10^6 cells/injection), B cells stimulated with 20 ng/ml IFNγ for 24 hours (2x10^6 cells/injection) or PBS. To discriminate between adoptively transferred cells and endogenous cells, B cells were isolated from CD45.1 mice for the last injection. Time between injections was 2 weeks. At the end of experiments, mice were sacrificed and relevant organs were harvested for analysis.

Cytokine analysis

Isolated splenocytes from PBS, B cell or IFNγ-B cell treated mice were cultured in complete RPMI medium and stimulated for 72 hours with anti-CD3 (1 μg/ml) and anti-CD28 (0.5 μg/ml). The levels of cytokines in culture supernatants were measured using a Luminex bead-based multiplex assay (ProcartaPlex, Thermo Fisher Scientific) on a Luminex Instrument (MAGPIX). Recombinant cytokine standards (Thermo Fisher Scientific) were used to calculate cytokine concentrations and data were analyzed using Bio-Rad software.

Flow cytometry

For flow cytometry analysis, Fc receptors of single cell suspensions were blocked with an unconjugated antibody against CD16/CD32. Samples were then stained with a fixable viability marker (ThermoScientific) to select live cells. Next, cells were stained with anti-mouse fluorochrome-conjugated antibodies (Supplementary table 2). Regular flow cytometry was performed on a Cytoflex S (Beckman Coulter) and

the acquired data were analyzed using FlowJo software (version 10). Gates were set according to isotype or fluorescence minus one controls.

Serum measurements

Serum was acquired by centrifugation and stored at -20°C until further use. Total serum titers of IgM, IgG1, IgG2c and oxidized LDL-specific antibodies were quantified by ELISA as previously described[7].

Histology

Carotid arteries and hearts were frozen in OCT compound (TissueTek) and stored at -80°C until further use. Transverse cryosections proximal to the collar were collected and mounted on Superfrost adhesion slides (ThermoFisher). To determine lesion size, cryosections were stained with hematoxylin and eosin (Sigma-Aldrich). Quantification of lesion size was assessed every 100 µm from the first section with visible lesion proximal to the collar until no lesion could be observed. Plaque volume was determined with lesion size and the total distance of the lesions in the carotid artery. Phenotypic analysis of the lesion was performed on sections containing the largest three lesions. Collagen content in the lesion was assessed with a Masson's trichrome staining according to the manufacturers protocol (Sigma-Aldrich). Corresponding sections on separate slides were also stained for monocyte/macrophage content using a monoclonal rat IgG2b antibody (MOMA-2, 1:1000, AbD Serotec) followed by a goat anti-rat IgG-horseradish peroxidase antibody (1:100, Sigma-Aldrich) and color development using the ImmPACT NovaRED substrate (Vector Laboratories). All slides were analyzed with a Leica DM-RE microscope and LeicaQwin software (Leica Imaging Systems).

Statistics

All data are expressed as mean ± SEM. Data were tested for significance using a Student's t-test for two normally distributed groups. Data from three groups or more were analyzed by an ordinary one-way ANOVA test followed by Holm-Sidak post hoc test. Probability values of $p < 0.05$ were considered significant. All statistical analyses were performed using GraphPad Prism 7.0.

Results

IFNγ-stimulated B cells express high levels of PD-L1

To investigate the role of PD-L1^{hi} B cells in atherosclerosis, we first aimed to generate PD-L1^{hi} B cells *ex vivo*. We explored several stimuli that previously have been

shown to control PD-L1 expression[18,19] (Fig. S1 and Fig 1) and found that IFNγ dose-dependently increased the number of PD-L1$^+$ B cells, with 20.0 ng/ml of IFNγ leading to an almost pure population of PD-L1-expressing B cells (Fig. 1A). Using qPCR, we also found an almost 8-fold induction of PD-L1 on mRNA level after IFNγ stimulation (Fig. 1B). Furthermore, we observed that the majority of IFNγ-stimulated B cells expressed very high levels of PD-L1 (Fig. 1C). Previously, it has been shown that IFNγ-signaling in B cells drives STAT-1 dependent expression of T-bet and BCL-6 and switches them towards a GC B cell phenotype with increased IFNγ and IL-6 production[20,21]. In line with this, our *ex vivo* IFNγ-stimulated B cells (IFNγ-B cells) showed a similar increase in STAT1, T-bet and BCL-6 (Fig 1D) gene expression. In contrast, we observed a trend towards less IL-6 and no difference in IFNγ expression (Fig 1D). Interestingly, IFNγ stimulation resulted in a strong significant increase in TGF-β expression (Fig. 1D). We also investigated the chemokine receptor profile of these B cells and found a dose-dependent increase in CCR7 expression, while there was no effect on CXCR5 or Ebi-2 (Fig. 1D). This change in chemokine receptor expression is typical of B cells that migrate towards the T-B cell border in lymphoid tissues in response to the CCL21 gradient[22]. Hence, these data indicate that IFNγ-stimulated B cells express high levels of PD-L1 and TGF-β and a chemokine profile that homes B cells to the T-B cell border.

IFNγ-stimulated B cells inhibit T_{FH} cells *in vitro* and *in vivo*

Next, we explored the functional effects of IFNγ-stimulated B cells on T_{FH} cell development using a CD4$^+$ T cell and B cell coculture. We stimulated wild-type CD4$^+$ T cells for 72 hours with anti-CD3 in the presence of unstimulated or IFNγ-stimulated *apoE*$^{-/-}$ B cells. Although we did not observe any difference in proliferative capacity of CD4$^+$ T cells (Fig. 2A), we found a remarkable decrease of T_{FH} cells when CD4$^+$ T cells were cocultured with IFNγ-stimulated B cells compared to unstimulated B cells (Fig 2B). These findings illustrate that IFNγ-B cells are able to curb T_{FH} cell development *in vitro*. We subsequently tested IFNγ-stimulated B cells in an *in vivo* setting by adoptively transferring either freshly isolated B cells or B cells stimulated with 20.0 ng/ml of IFNγ for 24 hours into *apoE*$^{-/-}$ mice. To induce initial T_{FH} cell accumulation, mice were fed a Western-type diet for two weeks before they received the adoptive transfers (Fig. 2C)[14]. We observed a remarkable reduction in effector CD4$^+$ T cells in mice treated with IFNγ-B cells compared to mice that received PBS or B cells (Fig. 2D). Contrary, the number of naïve CD4$^+$ T cells was increased in IFNγ-B cells treated mice compared to mice receiving PBS (Fig. 2D). Most importantly, treatment of mice with IFNγ-B cells resulted in a strong reduction in T_{FH} cells compared to mice that were administered with PBS or B cells (Fig. 2E). These data demonstrate that IFNγ-B cells are also able to inhibit T_{FH} cells *in vivo*.

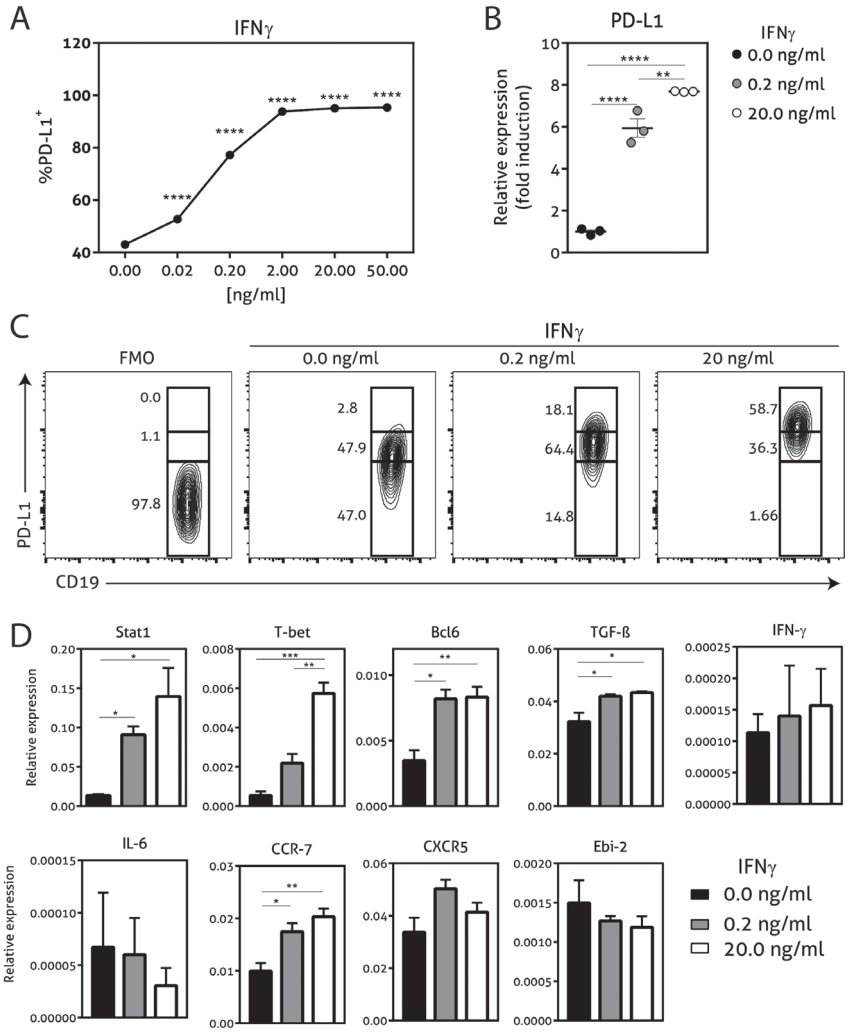


Figure 1. Characterization of IFNγ-stimulated B cells. CD19^{+} B cells were isolated from C57BL/6 mice and **(A)** stimulated for 24 hours with different doses interferon-gamma (IFNγ) after which PD-L1 protein expression was measured with flow cytometry. **(B)** CD19^{+} B cells were unstimulated or stimulated with 0.2 ng/ml or 20.0 ng/ml IFNγ for 24 hours, after which mRNA expression of PD-L1 was assessed using qPCR. **(C)** B cells as stimulated in (B) were analyzed for PD-L1^{lo}, PD-L1^{int} and PD-L1^{hi} expression with flow cytometry. **(D)** mRNA expression was analyzed for depicted genes in B cells as stimulated in (B). Data are analyzed with a One-Way ANOVA and shown as mean ± SEM (* p<0.05, ** p<0.01, ***p<0.001, ****p<.00001). n=3/group.

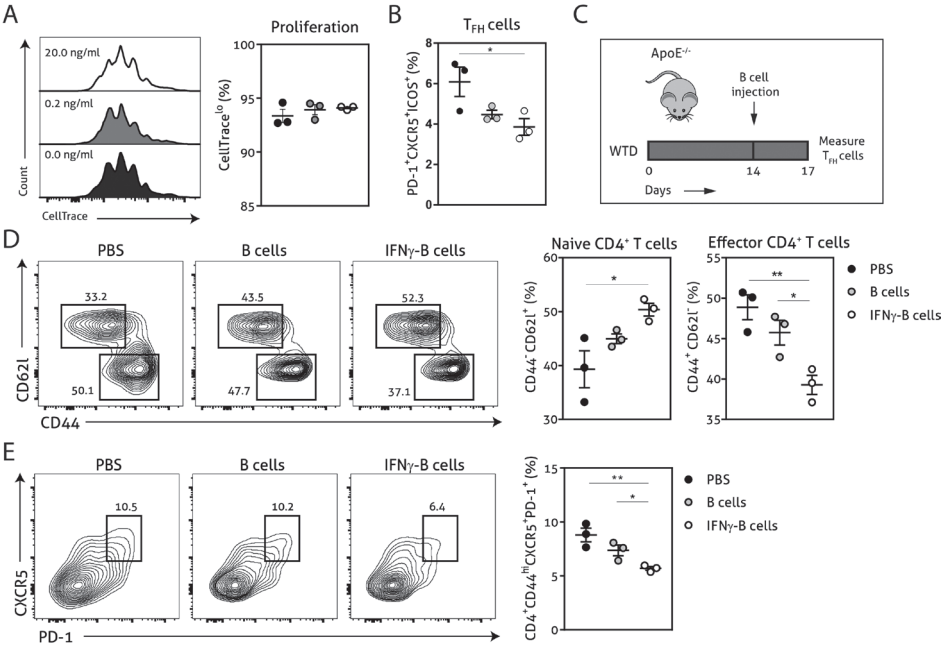


Figure 2. IFNγ-B cells inhibit T follicular helper cells *in vitro* and *in vivo*. Isolated CD19$^+$ B cells were unstimulated or stimulated with 0.2 ng/ml or 20.0 ng/ml IFNγ for 24 hours and co-cultured with isolated CD4$^+$ T cells. The coculture was stimulated with anti-CD3 (5 µg/ml) for 72 hours after which **(A)** proliferation of CD4$^+$ T cells was analyzed with CellTrace Violet and **(B)** the number of follicular CD4$^+$ T helper (T_{FH}) cells were analyzed. **(C)** *apoE*$^{-/-}$ mice were fed a Western type diet for 2 weeks after which they either received PBS, freshly isolated CD19$^+$ B cells (B cells) or CD19$^+$ B cells stimulated with 20.0 ng/ml IFNγ for 24 hours (IFNγ-B cells). After three days, mice were sacrificed and spleens were isolated for flow cytometry analysis for **(D)** naive (CD62l$^+$CD44$^-$) and effector (CD62l$^-$CD44^{hi}) CD4$^+$ T cells and **(E)** T follicular helper cells (CD4$^+$CD44^{hi}CXCR5$^+$PD-1$^+$). Representative flow charts of the CD4$^+$ T cell population are shown. Data are analyzed with a One-Way ANOVA and shown as mean ± SEM (* p<0.05, ** p<0.01). n=3/group.

Adoptive transfer of IFNγ-B cells during atherosclerosis development affects the T_{FH} – GC B cell axis

Given the *in vitro* and *in vivo* regulatory effects of IFNγ-B cells on T_{FH} cells, we further investigated whether these B cells would be able to restrict T_{FH} cell numbers during atherosclerosis development. We fed *apoE*$^{-/-}$ mice a Western-type diet for 2 weeks after which we placed a perivascular collar and started treatment with either PBS, B cells or IFNγ-B cells (Fig. 3A). After a total of 3 injections and 7 weeks of Western-type diet, we harvested the organs and analyzed the immune cells associated with the T_{FH} – GC B cell axis. IFNγ-B cell-treated mice showed a trend towards reduced FO B cells (Fig. 3B) and a significant increase in MZ B cells compared to mice receiving PBS (Fig. 3C). Moreover, mice that received IFNγ-B cells showed a significant increase in the number of GC B cells compared to mice that were administered

PBS (Fig 3D and E). By using the CD45 congenic marker system, we showed that adoptively transferred IFNγ-B cells indeed reached the germinal center (Fig. 3F) and locally increased the number of PD-L1^{hi} B cells (Fig. 3G). As shown in Fig. 3D and E, freshly isolated B cells also increased the number of GC B cells, but these were not derived from the adoptive transfer (Fig. 3F) and were PD-L1^{-} (Fig. 3H). At sacrifice, we did not observe a difference in T_{FH} cells in IFNγ-B cell-treated mice, while adoptive transfer of B cells resulted in an increase in T_{FH} cells compared to PBS-treated mice (Fig. 3I).

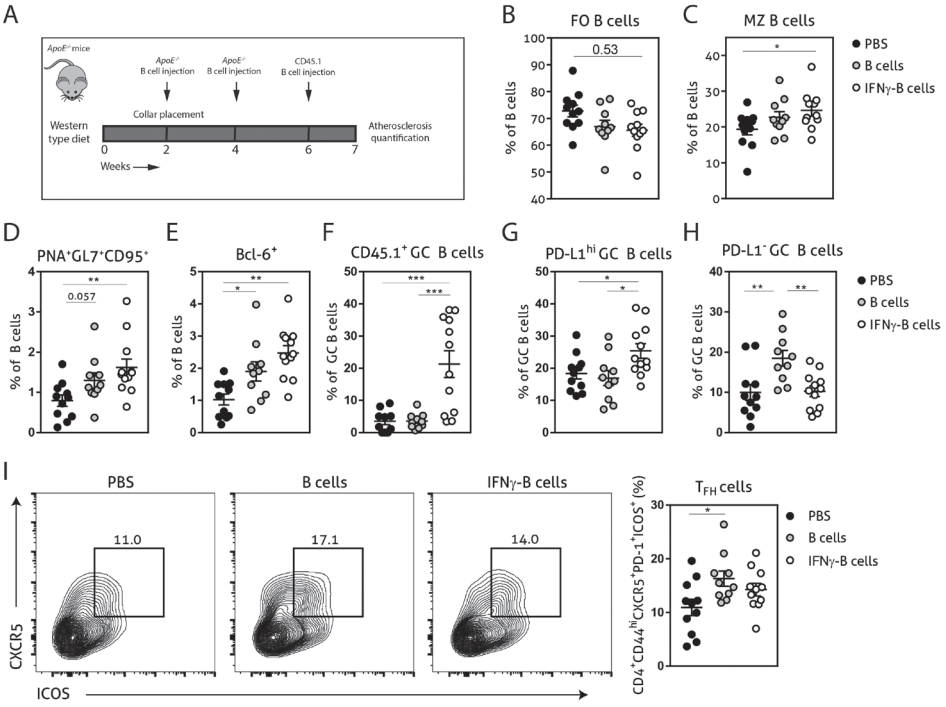


Figure 3. Adoptive transfer of IFNγ-B cells during atherosclerosis development affects the T_{FH} – GC B cell axis. (A) *ApoE*$^{-/-}$ mice were fed a Western type diet for 7 weeks. After 2 weeks they received a perivascular collar and were treated with PBS, freshly isolated B cells (B cells) or B cells stimulated with 20.0 ng/ml IFNγ for 24 hours (IFNγ-B cells). Mice received a total of three injections and injections were spaced every two weeks. After 7 weeks, mice were sacrificed and spleens were analyzed with flow cytometry. Flow cytometry quantification of **(B)** follicular (FO) B cells, **(C)** marginal zone (MZ) B cells, **(D)** extracellular staining of germinal center B cells, **(E)** intracellular staining of germinal center B cells, **(F)** CD45.1^{+} germinal center B cells, **(G)** PD-L1^{hi} germinal center B cells, **(H)** PD-L1^{-} germinal center B cells and **(I)** flow charts and quantification of follicular helper T cells (CD44^{hi}PD-1^{+}CXCR5^{+}ICOS^{+}). Data are analyzed with a One-Way ANOVA and shown as mean ± SEM (* p<0.05, ** p<0.01, *** p<0.001). n=10-12/group.

Next, we assessed the number of plasmablasts and plasma cells and found a significant reduction in plasmablasts when mice received IFNγ-B cells compared to mice receiving PBS (Fig. 4A). Mice that received B cells showed a similar trend towards less plasmablasts and also a significant increase in plasma cells compared to PBS- and IFNγ-B cells-treated mice. Since BLIMP-1 is the driving transcription factor for plasma cell generation[23], we also measured BLIMP-1 expression which revealed a significant increase in BLIMP-1^{+} cells in mice that received B cells compared to mice receiving PBS or IFNγ-B cells (Fig. 4B). Since plasmablasts and plasma cells are responsible for the humoral immunity, we measured circulating antibodies. However, neither B cell treatments led to a significant difference in circulating antibodies (Fig. 4C).

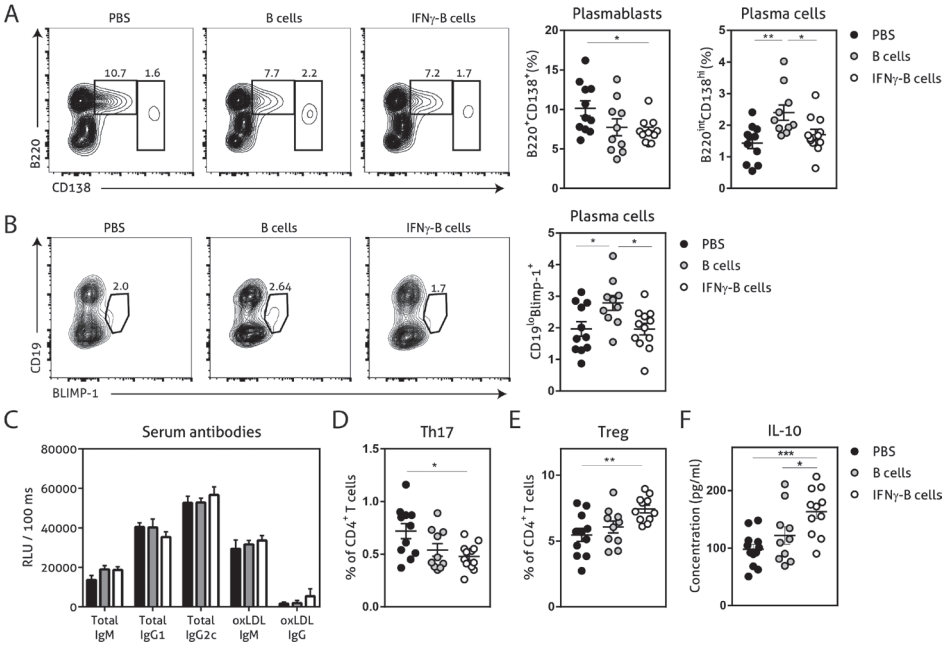


Figure 4. Effects of adoptive transfer of IFNγ-B cells on the humoral immunity. A*poE*$^{-/-}$ mice were fed a Western type diet for 7 weeks. After 2 weeks they received a perivascular collar and were treated with PBS, freshly isolated B cells (B cells) or B cells stimulated with 20.0 ng/ml IFNγ for 24 hours (IFNγ-B cells). Mice received a total of three injections and injections were spaced every two weeks. After 7 weeks, mice were sacrificed and spleens were analyzed with flow cytometry. **(A)** Flow charts and quantification of plasmablasts (B220^{+}CD138^{+}) and plasma cells (B220^{lo}CD138^{hi}). **(B)** Flow charts and quantification of CD19^{lo}BLIMP-1^{+} plasma cells. **(C)** Serum was analyzed for circulating antibodies by ELISA. Spleens were analyzed with flow cytometry for **(D)** Th17 cells (RORγt^{+}) or **(E)** Treg cells (FoxP3^{+}). **(F)** Splenocytes were isolated and stimulated with anti-CD3 (1 µg/ml) and anti-CD28 (0.5 µg/ml) for 72 hours after which supernatant was collected and analyzed for cytokine expression with a multiplex analysis. Quantification of IL-10 concentration is shown. Data are analyzed with a One-Way ANOVA and shown as mean ± SEM (* p<0.05, ** p<0.01). n=10-12/group.

Adoptive transfer of IFNγ-B cells promotes an anti-inflammatory $CD4^+$ T cell response

The $CD4^+$ T cell response is also highly involved in the pathogenesis of atherosclerosis and PD-$L1^{hi}$ B cells have previously shown to restrict $CD4^+$ T cell differentiation[17]. While we did not observe any differences in Th1 or Th2 $CD4^+$ T cells between mice treated with IFNγ-B cells, B cells or PBS in our study (Fig. S2), we observed a significant decrease in Th17 cells (Fig. 4D) and a significant increase in atheroprotective regulatory T cells (Fig. 4E; Tregs) in mice treated with IFNγ-B cells. We next measured cytokine levels of *ex vivo* anti-CD3 and anti-CD28 stimulated splenocytes for 72 hours with a multiplex assay. In line with the increase in Tregs, we observed a significant increase in IL-10 production by splenocytes from IFNγ-B cells treated mice compared to splenocytes from mice treated with PBS or B cells (Fig. 4F).

Adoptive transfer of IFNγ-B protects against lesion formation

Given the immune-regulating effects of IFNγ-B cells, we subsequently assessed whether adoptive transfer of these IFNγ-B cells was able to reduce collar-induced atherosclerosis in *apoE*$^{-/-}$ mice. We quantified lesion development in the carotid arteries and found a significant reduction in total lesion volume when mice were treated with IFNγ-B cells compared to PBS and a similar trend was found when compared to B cell-treated mice (Fig. 5A). Media size did not differ between the treatment groups (Fig. 5B). Furthermore, there were no differences in weight gain and serum cholesterol levels (Fig. S3A and S3B). We also assessed lesion phenotype at the site of the maximal lesion. This revealed that mice treated with IFNγ-B cells showed an early lesion phenotype with relatively more macrophages than collagen compared to mice that received B cells or PBS (Fig. 5C and 5D), suggesting that adoptive transfer of IFNγ-B cells inhibited atherosclerotic lesion progression towards more advanced lesions.

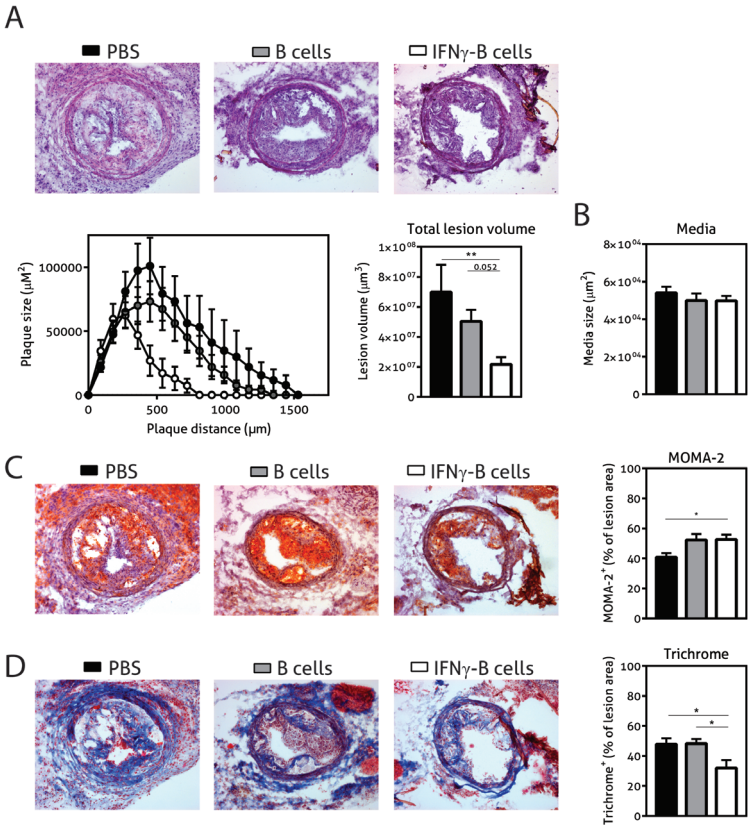


Figure 5. Adoptive transfer of IFNγ-B protects against atherosclerosis. *ApoE*$^{-/-}$ mice were fed a Western type diet for 7 weeks. After 2 weeks they received a perivascular collar and were treated with PBS, freshly isolated B cells (B cells) or B cells stimulated with 20.0 ng/ml IFNγ for 24 hours (IFNγ-B cells). Mice received a total of three injections and injections were spaced every two weeks. After 7 weeks, mice were sacrificed and **(A)** total lesion volume and **(B)** media size was determined in the right carotid artery with a hematoxylin and eosin staining. Lesion phenotype was determined in sections containing the largest lesions. **(C)** Macrophage content was analyzed using a MOMA-2 staining. **(D)** Collagen content was quantified with a Trichrome staining. Data are analyzed with a One-Way ANOVA and shown as mean ± SEM (* p<0.05, ** p<0.01). n=8-12/group.

Discussion

Up to date, three groups have reported the proatherogenic role of the T_{FH} – GC B cell axis[13–15]. Direct depletion of T_{FH} cells in *Ldlr*$^{-/-}$ mice resulted in a reduction of atherosclerosis[15], and loss of control on the T_{FH} – GC B axis by depletion of MZ B cells[14] or CD8^{+} regulatory T cells[13] aggravated atherosclerosis. We now show that adoptive transfer of PD-L1 expressing B cells inhibit T_{FH} cell responses both *in vitro* and *in vivo* and protect against atherosclerosis.

Using *ex vivo* stimulation of B cells with IFNγ we rapidly provided a large and almost pure population of PD-L1-expressing B cells. IFNγ is an extensively investigated cytokine with a broad range of immune actions. Interestingly, while we show here that IFNγ stimulation of B cells results in a regulatory phenotype that reduces the number of T_{FH} cells, earlier work identified a role for IFNγ in the generation of spontaneous germinal centers and B cell autoreactivity[20,24]. These previous data were, however, mainly acquired in the context of autoimmunity, where B cells receive a multitude of different signals and IFNγ signaling seems primarily to synergize with BCR-, CD40- and TLR-mediated stimuli to induce spontaneous germinal centers[24]. Under these circumstances, IFNγ signaling in B cells results in increased IL-6 and IFNγ production which drives auto-immunity[20,21]. In contrast, our *ex vivo* stimulated B cells lacked additional stimuli and upregulated expression of PD-L1 and TGF-β, while we did not see any effects on IL-6 and IFNγ. We further showed that IFNγ-B cells express high levels of CCR7 with minimal changes in CXCR5 and Ebi-2. This expression profile is in line with a previous study that demonstrated that MZ B cells from *Ldlr*$^{-/-}$ mice interact with pre-T_{FH} cells at the T-B cell border in response to a high-cholesterol diet[14]. During our atherosclerosis study, we indeed found that adoptively transferred IFNγ-B cells showed the characteristics of B cells that reside in or near the germinal center.

Similar to flow-sorted PD-L1^{hi} B cells[17], we next showed that IFNγ-B cells were able to inhibit T_{FH} cell numbers *in vitro* and *in vivo*. This study further demonstrated that IFNγ-B cells were able to promote anti-inflammatory CD4$^+$ T cells. This corresponds with the effects found in experimental autoimmune encephalomyelitis using PD-L1^{hi} B cells, which restricted Th1 and Th17 differentiation[17]. Along this line, we found that IFNγ-B cells resulted in decreased Th17 cells and a significant increase in atheroprotective Tregs and IL-10 production of CD4$^+$ T cells.

Furthermore, we demonstrated atheroprotective effects of IFNγ-B cells that express high levels of PD-L1, which is in line with a general protective role of the PD-L1/PD-1 axis in atherosclerosis. Mice deficient in both PD-L1 and PD-L2 show increased atherosclerosis[32]. Similarly, PD-1 knockout mice or mice treated with a PD-1 blocking antibody developed exacerbated atherosclerosis[33,34]. Our data is further supported by previous studies that demonstrated the proatherogenic role of T_{FH} cells[13–15]. However, due to the pleiotropic nature of IFNγ, other factors besides PD-L1 could also have contributed to our findings. Indeed, the observed increase in TGF-β expression could have contributed to both the T_{FH} inhibition and Treg induction, since TGF-β signaling is known to prevent T_{FH} cell accumulation and B cell autoreactivity[25]. In addition, there have been a large number of studies with adoptive

transfer of B cells expressing TGF-β that reported immune tolerance in autoimmune mouse models[26–29]. The majority of these studies reported Treg induction, which is in line with our findings of increased Tregs and IL-10 production after adoptive transfer of IFNγ-B cells. PD-L1 is also known to be essential for the induction of Treg cells[30], the Treg accumulation could thus be a combined effect of increased TGF-β and PD-L1 expression by IFNγ-B cells. Since the atheroprotective effects of Treg cells is well characterized[31], the observed Treg induction undoubtedly contributed to the reduced atherosclerosis found with adoptive transfer of IFNγ-B cells.

In conclusion, this study uncovers a new role for *ex vivo* stimulation of B cells with IFNγ for the induction of atheroprotective B cells. IFNγ-B cells show the genetic makeup of GC B cells with increased expression of PD-L1 and TGF-β and effectively inhibit T_{FH} cells *in vitro* and *in vivo* and ameliorate atherosclerosis development in *apoE*$^{-/-}$ mice. These results further emphasize the proatherogenic role of the T_{FH} – GC B axis and provide a novel way to regulate this axis.

Acknowledgements

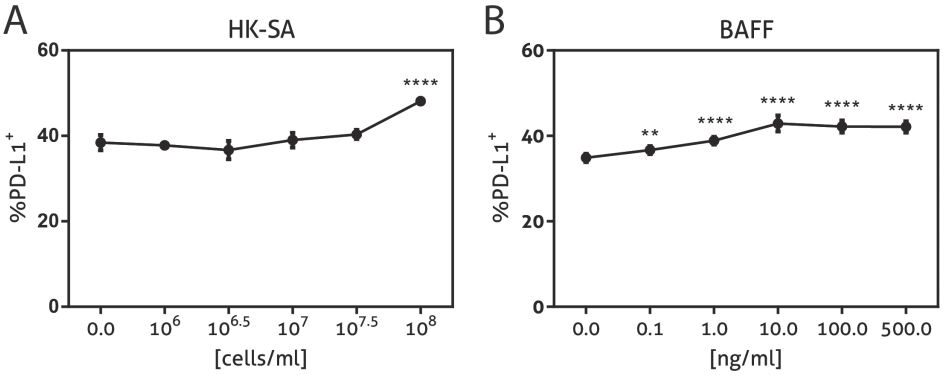
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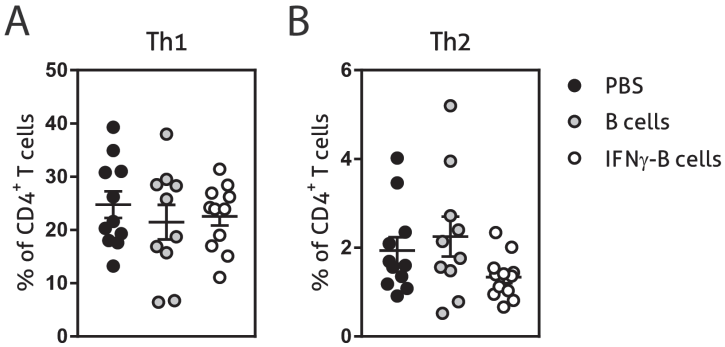
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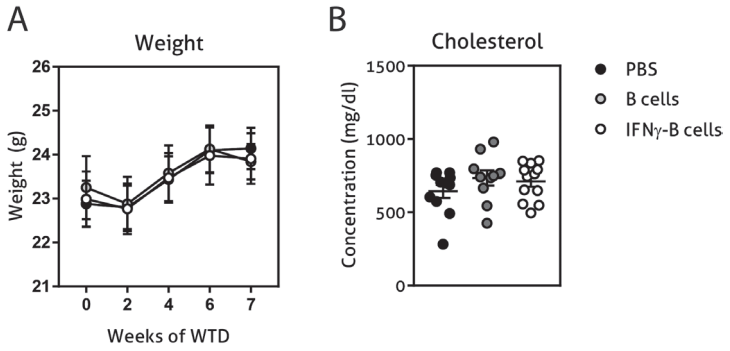
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Supplementary Figure 1. PD-L1 expression of stimulated B cells. CD19+ B cells were isolated from C57BL/6 mice and stimulated for 24 hours with different doses of (A) heat killed S. Aureus (HK-SA) or (B) B cell activating factor (BAFF) after which PD-L1 expression was measured with flow cytometry. Data are analyzed with a One-Way ANOVA and shown as mean ± SEM (** p<0.01, ****p<.00001). n=3/group.



Supplementary Figure 2. **Effects of adoptive transfer of IFNγ-B cells on Th1 and Th2 cells.** *ApoE*$^{-/-}$ mice were fed a Western type diet for 7 weeks. After 2 weeks they received a perivascular collar and were treated with PBS, freshly isolated B cells (B cells) or B cells stimulated with 20.0 ng/ml IFNγ for 24 hours (IFNγ-B cells). Mice received a total of three injections and injections were spaced every two weeks. After 7 weeks, mice were sacrificed and spleens were analyzed with flow cytometry for **(A)** Th1 cells (T-bet^{+}) and **(B)** Th2 cells (Gata-3^{+}). Data are shown as mean ± SEM. n=10-12/group.



Supplementary Figure 3. *ApoE*$^{-/-}$ mice were fed a Western type diet for 7 weeks. After 2 weeks they received a perivascular collar and were treated with PBS, freshly isolated B cells (B cells) or B cells stimulated with 20.0 ng/ml IFNγ for 24 hours (IFNγ-B cells). Mice received a total of three injections and injections were spaced every two weeks. **(A)**Weight was assessed during the experiment and **(B)** and cholesterol serum levels were analyzed at the end of the experiment.

Table 1

Target	Accession #	Forward primer	Reverse primer
CD274 (CD274 antigen, PD-L1)	NM_021893	ggactacaagcgaatcacgctg	tcatgctcagaagtggctggat
Tbx21 (T-box 21, Tbet)	NM_019507	agtgactgcctaccagaacgcagag	ccaggtggcgaggggacact
Bcl6 (B cell leukemia/lymphoma 6)	NM_009744	ctgcaacgaatgtgactgccgtttc	ccgattgaactgcgctccacaaatg
Il6 (interleukin 6)	NM_031168	agacaaagccagagtccttcagaga	ggagagcattggaaattggggtagg
infg (interferon gamma)	NM_008337	ccttcttcagcaacagcaaggcga	gcgctggacctgtgggttgt
TGF-(b) (transforming growth factor, beta 1, Tgfb1)	NM_011577	agggctaccatgccaacttct	gcaaggaccttgctgtactgtgt
CCR7 (chemokine (C-C motif) receptor 7, EBI-1)	NM_007719	cgtgctggtggtggctctcct	accgtggtattctcgccgatgtagtc
Cxcr5 (chemokine (C-X-C motif) receptor 5)	NM_007551	cgagctgagtggctatctctct	agaggtcactgcggaactttac
Gpr183 (G protein-coupled receptor 183; EBI-2)	NM_183031	ggacgcctgctcgatcactcttaaa	acgttgccagtggggtagtgaaatt
B-actin (actin, beta, Actb)	NM_007393	cttctttgcagctccttcgttgccg	aatacagcccggggagcatcgtc
Rpl27 (Ribosomal protein L27)	NM_011289	cgccaagcgatccaagatcaagtcc	agctgggtccctgaacacatccttg
Rplp0 (ribosomal protein, large, P0, 36B4)	NM_007475	ctgagtacaccttcccacttactga	cgactcttcctttgcttcagcttt

Table 2

Antigen	Clone	Fluorochrome	Supplier
B220	RA3-6B2	PerCP-Cy5.5	ThermoFisher
B220	RA3-6B2	Alexa fluor 700	ThermoFisher
BCL-6	K112-91	Brilliant Violet 421	BD Biosciences
Blimp-1	5E7	Alexa fluor 647	Biolegend
CD138	281-2	Brilliant Violet 421	Biolegend
CD19	6D5	Brilliant Violet 605	Biolegend
CD21	7G6	Brilliant Violet 421	Biolegend
CD23	B3B4	PE	ThermoFisher
CD279 (PD1)	J43	FITC	ThermoFisher
CD4	RM4-5	FITC	ThermoFisher
CD4	RM4-5	PerCP	ThermoFisher
CD44	IM7	APC	ThermoFisher
CD44	IM7	BV510	Biolegend
CD45.1	A20	PE	ThermoFisher
CD5	53-7.3	FITC	BD Biosciences
CD62L	MEL-14	FITC	Biolegend
CD8a	53-6.7	FITC	ThermoFisher
CD8a	53-6.7	Alexa fluor 700	Biolegend
CD93	AA4.1	PE-Cy7	ThermoFisher
CD95	Jo2	PE-Cy7	ThermoFisher
CXCR5	L138D7	PE	Biolegend
Fixable Viability Dye		APC-efluor 780	ThermoFisher
FoxP3	FJK-16s	Pacific Blue	ThermoFisher
Gata-3	TWAJ	PE	ThermoFisher
GL7	GL-7	efluor 450	ThermoFisher
ICOS	7E.17G9	PE-Cy7	ThermoFisher
IgD	11-26	APC	ThermoFisher
NK1.1	PK136	FITC	BD Biosciences
PD-L1	10F.9G2	PE-Dazzle 594	Biolegend
PNA		FITC	Vectorlabs
RORyt	Q31-378	Brilliant Violet 650	BD Biosciences
T-bet	eBio4B10	PE-Cy7	ThermoFisher

BTLA stimulation protects against atherosclerosis by regulating follicular B cells

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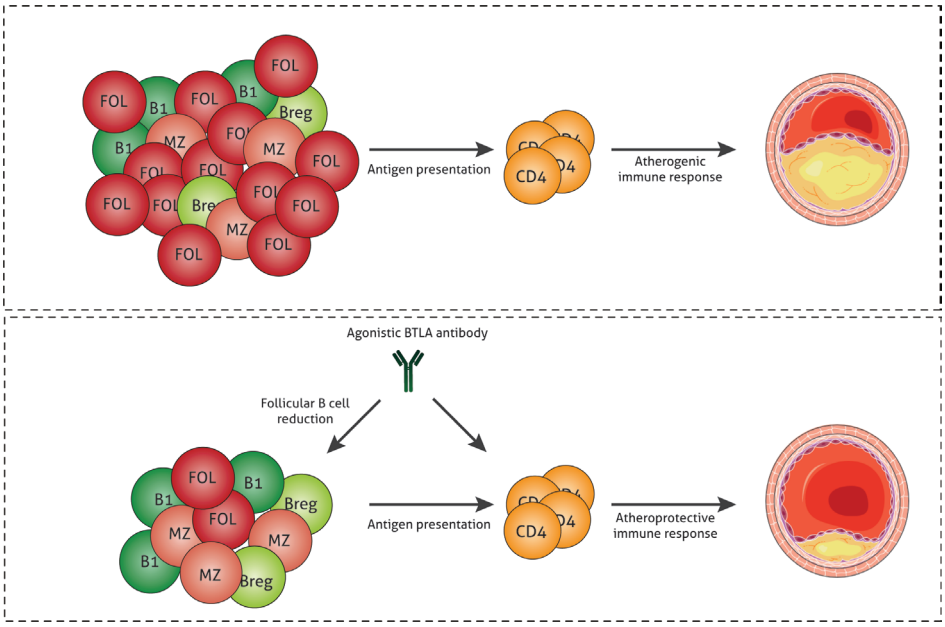
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Abstract

Aims: The immune system is strongly involved in atherosclerosis and immune regulation generally leads to attenuated atherosclerosis. B and T lymphocyte attenuator (BTLA) is a novel co-receptor that negatively regulates the activation of B and T cells, however, there have been no reports of BTLA and its function in atherosclerosis or cardiovascular disease (CVD). We aimed to assess the dominant BTLA expressing leukocyte in CVD patients and to investigate whether BTLA has a functional role in experimental atherosclerosis.

Methods and results: We show that BTLA is primarily expressed on B cells in CVD patients and follicular B2 cells in low-density lipoprotein receptor-deficient (*Ldlr*$^{-/-}$) mice. We treated *Ldlr*$^{-/-}$ mice that were fed a Western-type diet (WTD) with PBS, an isotype antibody or an agonistic BTLA antibody (3C10) for 6 weeks. We report here that the agonistic BTLA antibody significantly attenuated atherosclerosis. This was associated with a strong reduction in follicular B2 cells, while regulatory B and T cells were increased. The BTLA antibody showed similar immunomodulating effects in a progression study in which *Ldlr*$^{-/-}$ mice were fed a WTD for 10 weeks before receiving antibody treatment. Most importantly, BTLA stimulation stabilized preexisting lesions.

Conclusion: Stimulation of the BTLA pathway in *Ldlr*$^{-/-}$ mice reduces initial lesion development and increases stability of established lesions, presumably by shifting the balance between atherogenic follicular B cells and atheroprotective B cells and directing CD4^{+} T cells towards regulatory T cells. We provide the first evidence that BTLA is a very promising target for the treatment of atherosclerosis.



Graphical abstract.

Introduction

Inflammation is a key process in the development of atherosclerosis[1]. Components of both the innate and adaptive immune system contribute significantly to the pathology of atherosclerosis. The connection between these two arms of the immune system is provided by professional antigen presenting cells (APCs). Dendritic cells and macrophages are well known APCs, but nowadays the role of B cells as potent APCs is also increasingly recognized. Next to processing and presenting antigens to lymphocytes, these cells provide additional stimuli in the form of co-receptors. In fact, the activation of T cells and B cells is tightly controlled by these immune checkpoint proteins [2]. APCs have a wide-ranging variety of both stimulatory and inhibitory immune checkpoint proteins. In general, stimulatory co-receptors are found to be atherogenic, while inhibitory proteins attenuate atherosclerosis [2].

B- and T-lymphocyte attenuator (BTLA, also known as CD272) is a relatively recently described co-inhibitory receptor belonging to the immunoglobulin superfamily. It was originally identified in search for a T helper type 1 (Th1) marker [3] and it has been demonstrated that BTLA interacts with herpesvirus entry mediator (HVEM) [4]. HVEM is part of the tumor necrosis factor receptor superfamily and the BTLA-HVEM interaction is an unique example of cross-talk between the immune

checkpoint proteins of both superfamilies. Ligation of HVEM to BTLA generates an inhibitory signal which is supported by the fact that BTLA-deficient T and B cells are hyperresponsive [3,5,6]. In addition, BTLA-deficient mice show a marked phenotype of auto-immunity with increased levels of auto-antibodies, spontaneous development of autoimmune-like hepatitis [7] and increased susceptibility towards experimental autoimmune encephalomyelitis [3]. In humans, BTLA seems to function similarly since BTLA cross-linking on human T cells leads to suppressed proliferation and cytokine production [8]. Moreover, in patients with rheumatoid arthritis [9] and type 1 diabetes [10] functional polymorphisms or altered expression levels of BTLA have been found.

Hence, since its discovery as an inhibitory co-receptor, BTLA has attracted a lot of attention as a potential target for immunotherapy. While in oncology novel ways to block BTLA are explored [11], it has been shown that in autoimmune or inflammatory disorders stimulation of BTLA is a more promising option. For instance, agonistic antibodies for BTLA have been used in experimental models of Behcet's disease [12], cerebral malaria [13], graft-versus-host disease [14] and cardiac allograft rejection [15]. These data clearly indicate that the BTLA pathway is a potent option for the treatment of autoimmune disorders. Up to date there have been no reports of BTLA and if it is involved in atherosclerosis or CVD.

In the present study, we therefore aimed to characterize BTLA-expressing leukocytes in CVD patients and to investigate whether BTLA has a functional role in atherosclerosis by using a non-depleting agonistic BTLA antibody.

Methods

Human samples

Eleven anonymous atherosclerotic plaques and blood were collected post-operatively from carotid or femoral arteries endarterectomy surgeries performed in 2017 and 2018 at the Haaglanden Medical Center (HMC), Westeinde, The Hague, The Netherlands. The collection of samples was performed conform the declaration of Helsinki regarding ethical principles for medical research involving human subjects (METC registration number 17-046). All atherosclerotic samples were processed to single cell suspensions as described previously[16]. In short, cell suspensions from human atherosclerotic plaques were obtained upon digestion with collagenase IV (Gibco) and DNase (Sigma) for 30 minutes at 37°C prior to single-cell separation through a 70µm cell strainer. Red blood cells in blood samples were lysed using ACK lysis buffer. All human white blood cell populations were characterized by

flow cytometry based on the expression of the pan-leukocyte marker CD45. Gating strategies are shown where appropriate.

Animals

Female low-density lipoprotein receptor-deficient mice (*Ldlr*$^{-/-}$) were bred in house and kept under standard laboratory conditions. Mice were fed a Western-type diet (WTD) containing 0.25% cholesterol and 15% cocoa butter (Special Diet Services, Witham, Essex, UK). Diet and water were provided ad libitum. All injections were administered i.p. in a total volume of 100 µl. During the experiments, mice were weighed, and blood samples were obtained by tail vein bleeding. At the end of experiments, mice were anaesthetized by a subcutaneous injection of a cocktail containing ketamine (40 mg/mL), atropine (50 µg/mL), and sedazine (6.25 mg/mL). Mice were bled by femoral artery transection followed by perfusion with phosphate-buffered saline (PBS) through the left cardiac ventricle. All animal work was approved by the Leiden University Animal Ethics Committee and the animal experiments were performed conform the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

In vivo experiments

Two separate diet-induced atherosclerosis experiments were performed in this study; an atherosclerosis initiation and an atherosclerosis progression experiment. For the initiation experiment, female *Ldlr*$^{-/-}$ mice (n=11-12/group) were fed a WTD for 6 weeks while either receiving an i.p. injection of PBS, an appropriate Armenian hamster isotype antibody (Innovative Research, Novi, MI; 100 µg/injection) or a non-depleting agonistic BTLA antibody[15] (3C10; 100 µg/injection) twice a week. For the progression experiment, female *Ldlr*$^{-/-}$ mice (n=11-12/group) were first fed a WTD for 10 weeks before initiating the same treatment with an isotype antibody or an agonistic BTLA antibody for 6 weeks. At the start of the treatment, one group of mice was sacrificed as a baseline group. A third in vivo experiment was performed to investigate the direct effect of BTLA blockade on T cells. Female *Ldlr*$^{-/-}$ mice (n=5-6/group) were fed a WTD for 2 weeks while, similarly as described above, receiving i.p. injections of either PBS, isotype control, BTLA, or receiving once a week anti-CD20 (Genentech, clone 5D2; 250 µg/injection) to deplete B cells, or a combination of BTLA and anti-CD20.

Flow cytometry

For flow cytometry analysis, Fc receptors of single cell suspensions of the mediastinal lymph node, spleen, blood or peritoneum were blocked with an unconjugated antibody against CD16/CD32. Samples were then stained with a fixable viability marker

(ThermoScientific) to select live cells and anti-mouse fluorochrome-conjugated antibodies (see Online Supplementary Table 1).

For the analysis of IL-10$^+$ B cells, single cell suspensions were stimulated for 5 hours with LPS (50 µg/ml), PMA (50 ng/ml), ionomycin (500 ng/ml) and monensin (2 µM). FACS analysis was performed on a FACSCanto II (Becton Dickinson) or a Cytoflex S (Beckman Coulter) and the acquired data were analyzed using FlowJo software. Gates were set according to unstimulated controls (only treated with monensin) or to isotype and fluorescence minus one controls.

Serum measurements

After euthanasia, orbital blood was collected in EDTA-coated tubes. Whole blood cell counts were analyzed using the XT-2000iV hematology analyzer (Sysmex Europe GMBH, Norderstedt, Germany). Serum was acquired by centrifugation and stored at −20°C until further use. The total cholesterol levels in serum were determined using enzymatic colorimetric procedures (Roche/Hitachi, Mannheim, Germany). Precipath (Roche/Hitachi) was used as an internal standard. Total serum titers of IgM, IgG1, IgG2c and antigen-specific antibodies were quantified by ELISA as previously described [17].

B cell culture

B cells were isolated from female *Ldlr*$^{-/-}$ mice using CD19$^+$ microbeads (Miltenyi Biotec) and cultured in complete RPMI medium in the presence of different concentrations of the agonistic BTLA antibody or recombinant murine HVEM Fc (R&D systems). After 24 hours cells were harvested and either stained using an Annexin-V Apoptosis detection kit (ThermoFisher) or used for routine flow cytometry.

B cells were also isolated from mice treated with PBS or the BTLA agonistic antibody for 2 weeks. Isolated B cells were co-cultured with isolated CD4$^+$ T cells from OT-II mice in the presence of OVA323 peptide (1 µg/ml). After 72 hours, cells were harvested and analyzed with flow cytometry.

Histology

To determine plaque size, cryosections (10 µm) of the aortic root were stained with Oil-Red-O and hematoxylin (Sigma-Aldrich). Sections with the largest lesion plus four flanking sections were analyzed for lesion size and two flanking sections for lesion composition. Collagen content in the lesion was assessed with a Masson's trichrome staining according to the manufacturers protocol (Sigma-Aldrich). Corresponding sections on separate slides were also stained for monocyte/macrophage with a

MOMA-2 antibody (1:1000, AbD Serotec) followed by a goat anti-rat IgG-alkaline phosphatase antibody (1:100, Sigma-Aldrich). Color development was achieved using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as enzyme substrates. The relative amount of collagen and percentage of macrophages in the lesions is expressed as the ratio between the collagen- or MOMA-2-positive and the total lesion surface area. Smooth muscle cells were stained with an α-smooth muscle cell-actin (αSMA) antibody (clone 1A4, Sigma-Aldrich) and SMC positive areas were related to the total intima surface area. The necrotic core was defined as the a-cellular, debris-rich plaque area as percentage of total plaque area. $CD4^+$ T cell (RM4-5, 1:90, ThermoFisher) and B cell (RA3-6B2, 1:100, BD Biosciences) analysis in the lesion was assessed using a rat monoclonal antibody and a secondary rabbit anti-rat antibody (BA-4001, Vector). Followed by the Vectastain ABC kit (PK-4000, Vector) and color was developed using the ImmPact Novared kit (Vector). Sections where no primary antibody was used were taken as negative controls. All slides were analyzed with a Leica DM-RE microscope and LeicaQwin software (Leica Imaging Systems).

For fluorescent histology, cryosections (10 µm) of the spleen were stained with anti-mouse antibodies against CD3 (SP7) and B220 (RA3-6B2). Subsequently, sections were stained with secondary goat-anti rabbit and goat-anti rat antibodies conjugated to Alexa fluor 647 and Alexa fluor 488 (Abcam). Nuclei were visualized using the Fluoroshield mounting medium containing DAPI. Sections were captured using a Nikon TiE 2000 confocal microscope with a 20x plan apochromat objective and analyzed with Nis Elements version 4.3.

Statistics

All data are expressed as mean ± SEM. Data were tested for significance using a Student's t-test for two normally distributed groups. Data from three groups or more were analyzed by an ordinary one-way ANOVA test followed by a Holm-Sidak post hoc test. Data from experiments with 2 or more variables were analyzed by a two-way ANOVA test followed by a Sidak post hoc test. Probability values of $p < 0.05$ were considered significant. All statistical analysis was performed using GraphPad Prism 7.0.

Results

B cells from CVD patients display strong BTLA expression

There is currently no data available regarding BTLA in patients with CVD. Hence, we obtained blood and lesions from patients that underwent an endarterectomy to assess the expression of BTLA on leukocytes. We found that almost 90% of the circulating $CD19^{+}CD20^{+}$ B cells expressed high levels of BTLA, while $CD3^{+}$ T cells (8.5%) and monocytes (5.2%) showed a much more moderate expression and granulocytes expressed only minimal levels of BTLA (Online Figure I, Figure 1A and B). Within the lesion, the greater majority of leukocytes, including B cells, do not express BTLA. This is partly caused by a difference in B cell subsets that reside in the lesion compared to blood (Figure 1B). The circulating B cell population is mostly composed of naïve B cells that express the highest levels of BTLA, whereas in the lesion we mainly found effector B cell subtypes that have lower BTLA expression. These data suggest that BTLA is most abundantly expressed on circulating B cells in patients with CVD, while this expression seems to be lost on B cells that are present in the lesion.

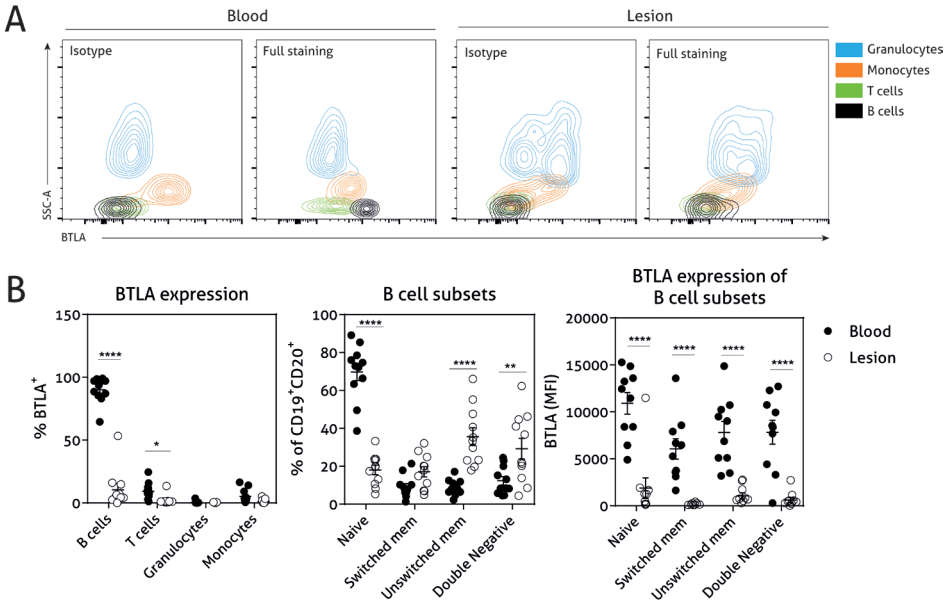


Figure 1. B cells from CVD patients display strong BTLA expression

Flow cytometry was applied on blood and lesions from CVD patients to identify BTLA expression on major leukocyte populations. **(A)** Flow charts of BTLA expression by major leukocyte populations. **(B)** Quantification of BTLA expression by leukocyte populations and B cell subsets. A two-way ANOVA followed by a Sidak post hoc test was performed. Data are shown as mean ± SEM, n=11 (* $p<0.05$, ***$p<0.001$, **** $p<0.0001$ vs. isotype).

BTLA is predominantly expressed by follicular B2 cells in *Ldlr*$^{-/-}$ mice

Given the high expression of BTLA on peripheral B cells from CVD patients and its inhibitory role, we wanted to further explore the potential of BTLA as a therapeutic target to treat atherosclerosis. First, we characterized the expression profile of BTLA on immune cells using spleens from low-density lipoprotein receptor-deficient (*Ldlr*$^{-/-}$) mice. Similar to human leukocytes, we found that BTLA was predominantly expressed on B cells, with relatively low expression on CD4^{+} T cells, CD8^{+} T cells and innate immune cells (Figure 2A and Online Figure II-A). Moreover, we did not find any differences in BTLA expression between CD4^{+} T helper cell subsets in contrast to previous work [3] (Online Figure II-B). Interestingly, we again discovered a significant difference in BTLA expression between B cell subsets, with conventional follicular B2 cells being the most dominant BTLA-expressing leukocyte (Figure 2B).

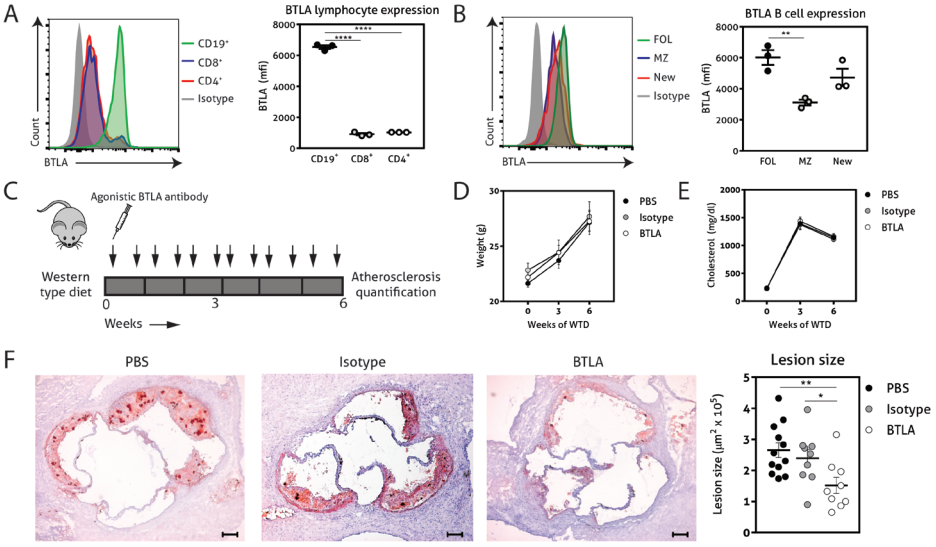


Figure 2. Activation of BTLA reduces initial atherosclerosis.
(A) Flow overlays and quantification of BTLA expression in leukocyte populations and **(B)** specific B cell subsets of female *Ldlr*$^{-/-}$ mice; follicular (CD21^{int}CD23^{+}); marginal zone (CD21^{hi}CD23^{-}) and newly formed (CD23^{lo}CD21^{lo}). **(C)** Female *Ldlr*$^{-/-}$ mice were treated twice a week intraperitoneally with PBS, an isotype antibody or an agonistic BTLA antibody for 6 weeks while being fed a Western-type diet. **(D)** Mice were weighed and **(E)** serum cholesterol levels were determined. **(F)** Oil-Red-O and hematoxylin staining in aortic root sections and lesion size quantification. Scale bars are 100 µm. An ordinary one-way ANOVA test followed by a Holm-Sidak post hoc test was performed. Data are shown as mean ± SEM, n=3 (A/B) and n=9/12 (C-F) (** p<0.01, **** p<0.0001).

BTLA activation reduces initial atherosclerosis

The role of B cells in atherosclerosis has been quite controversial and seems to be highly dependent on the B cell subset [18]. Previously, it has been reported that

follicular B cells aggravate atherosclerosis [19–21]. Since BTLA was most abundantly expressed on follicular B cells, we hypothesized that an agonistic BTLA antibody could inhibit follicular B cells in *Ldlr*$^{-/-}$ mice, leading to attenuated atherosclerosis. We therefore treated female *Ldlr*$^{-/-}$ mice twice a week with either PBS, an isotype control antibody or an agonistic BTLA antibody (3C10) for 6 weeks while being fed a Western-type diet (WTD) (Figure 2C). The 3C10 antibody has previously been described as a non-depleting agonistic BTLA antibody [15]. Treatment with the BTLA antibody did not affect body weight or total serum cholesterol levels (Figure 2D and E). Stimulation/agonism of BTLA resulted in a significant 43% reduction in lesion size in the aortic root compared to PBS-treated mice and a significant 37% reduction compared to isotype-treated mice (Figure 2F). Correspondingly, we found that lesions from mice treated with the BTLA antibody predominantly consisted of macrophages and relatively low amounts of collagen (Online Figure III), illustrating an early lesion phenotype. In contrast, lesions from mice treated with PBS or the isotype control, showed a more advanced phenotype with relatively more collagen and less macrophage content (Online Figure III).

Activation of BTLA leads to strong follicular B2 cell reduction

To assess whether the reduced atherosclerosis could be attributed to altered immune functions, we analyzed circulating leukocytes. We found a major reduction in total white blood cells in mice treated with the BTLA antibody compared to PBS- or isotype-treated mice (Online Figure IV). This was primarily due to a major decrease in lymphocytes and a smaller decrease in monocytes. Since monocytes and macrophages contribute significantly to initial lesion formation, we measured monocytes and monocyte subsets with flow cytometry but we could not identify a difference between BTLA-treated mice and PBS- or isotype-treated mice (Online Figure V). The strong lymphocyte reduction was primarily due to a sharp decrease in B cells in the blood and a similar effect was found in the spleen, lymph node and peritoneum (Figure 3A, Online Figure VI-A). Moreover, analysis of the B cell lineages showed that BTLA agonism particularly led to a reduction in B2 cells and a relative increase in the percentage of B1 cells (Figure 3B, Online Figure VI-B). More specifically, we found that the percentage of follicular B2 cells decreased, while marginal zone B cells increased in mice treated with the BTLA antibody compared to mice treated with PBS or isotype control (Figure 3C, Online Figure VI-C). In line with these findings, fluorescent immunohistology on spleen cryosections revealed that BTLA stimulation led to a decrease of B cells immediately surrounding the T cell areas, which corresponds to a reduction in follicular B cells (Figure 3D).

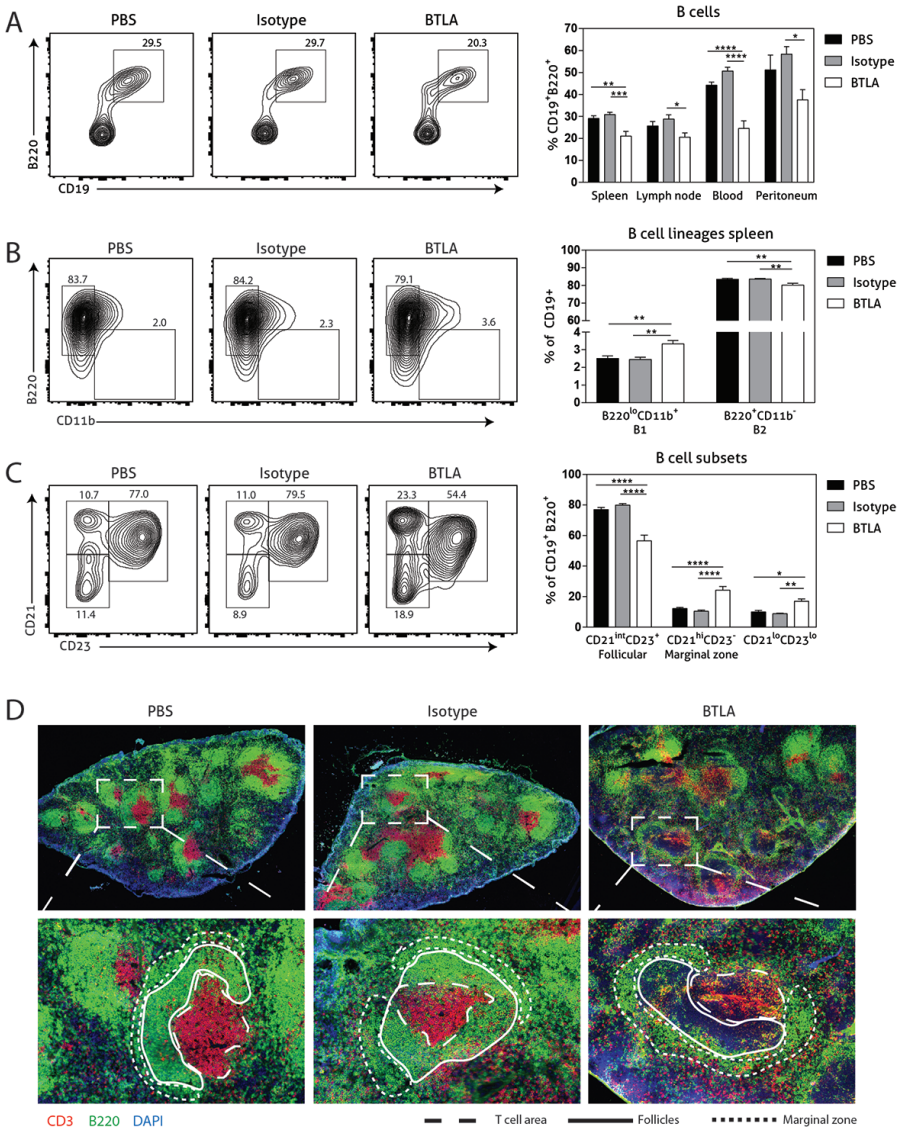


Figure 3. Activation of BTLA leads to strong follicular B2 cell reduction.
Female *Ldlr*$^{-/-}$ mice were treated twice a week with PBS, an isotype antibody or an agonistic BTLA antibody and fed a WTD for 6 weeks. **(A)** Flow charts of splenocytes and quantifications of B cells ($CD19^+B220^+$) in different organs. **(B)** Flow charts of splenocytes and quantification of B1 cells ($CD19^+CD11b^+B220^{lo}$) and B2 cells ($CD19^+CD11b^-B220^+$) in the spleen. **(C)** Flow charts of splenocytes and quantification of follicular ($CD21^{int}CD23^+$), marginal zone ($CD21^{hi}CD23^-$) and newly formed ($CD23^{lo}CD21^{lo}$) B cells in the spleen. **(D)** Representative images of spleen sections stained for CD3 (red), B220 (green) and DAPI (blue). Regions of interest are visualized as depicted at higher magnifications. Scale bars are 100 µm and 500 µm. An ordinary one-way ANOVA test followed by a Holm-Sidak post hoc test was performed. Data are shown as mean ± SEM, n=11-12 (*p<0.05, ** p<0.01, ***p<0.001, **** p<0.0001).

Mechanistically, we show that BTLA agonism, similarly as BTLA-HVEM signaling, increased apoptosis and reduced activation in follicular B cells (Online Figure VII). Since follicular B cells are often involved in the humoral immunity, we next determined whether BTLA treatment reduced atherosclerosis by altering antibody responses. However, we did not find relevant differences in either total or malondialdehyde-LDL and oxidized-LDL-specific serum titer levels that could explain the ameliorated atherosclerosis (Online Figure VIII).

Activation of BTLA leads to increased regulatory B cells

Besides the conventional B1 and B2 cells, we nowadays know that there are many novel B cell subsets that can regulate the immune response [18]. Although most of these regulatory B cells (Bregs) ultimately act via interleukin-10 (IL-10), they can be identified by different extracellular markers [22], such as $CD1d^{hi}CD5^{+}$ expression [23], and T-cell immunoglobulin and mucin domain 1 (TIM-1) [24]. We found that BTLA was expressed on all of these Breg subsets (Online Figure II-C). Accordingly, *Ldlr*$^{-/-}$ mice treated with agonstic BTLA showed a very strong increase in B10 cells and TIM-1^{+} B cells in the spleen, lymph nodes and blood when compared to treatment with PBS or isotype control (Figure 4A and B). In addition, we measured the direct secretion of IL-10 by B cells and found that BTLA stimulation significantly increased the percentage of B cells that secreted IL-10 compared to the PBS or isotype control groups (Figure 4C).

Activation of BTLA leads to a protective T cell response

Treatment with the BTLA antibody resulted in a B cell pool that is highly enriched in marginal zone B cells and Breg cells, which can both inhibit the $CD4^{+}$ T cell response [22,25]. Hence, we assessed the number of lesional $CD4^{+}$ T cells and found a marked reduction in infiltrating $CD4^{+}$ T cells in BTLA-treated mice compared to PBS- or isotype-treated mice (Figure 5A). Corroborating with earlier data [21], we did not find any relevant numbers of B cells in the lesion at this stage (Online Figure IX). We thus reasoned that the $CD4^{+}$ T cell regulation had taken place peripherally as shown in earlier reports [21]. Although we did not find a difference in total splenic $CD4^{+}$ T cells, we found that BTLA-treated mice contained more effector and less naïve T cells than PBS- or isotype-treated mice (Figure 5B). More specifically, we observed an increase in regulatory (Treg) and Th17 cells in BTLA-treated mice, while no effects were found for Th1 or Th2 cells (Figure 5C). It is widely recognized that Tregs are atheroprotective [26] and Th17 cells have also been shown to protect against atherosclerosis in a setting with reduced B cells [21]. This indicates that treatment with the BTLA antibody polarized the $CD4^{+}$ T cell response *in vivo* towards a more atheroprotective reponse. The reduced activation of $CD4^{+}$ T cells in BTLA-treated mice was not dependent

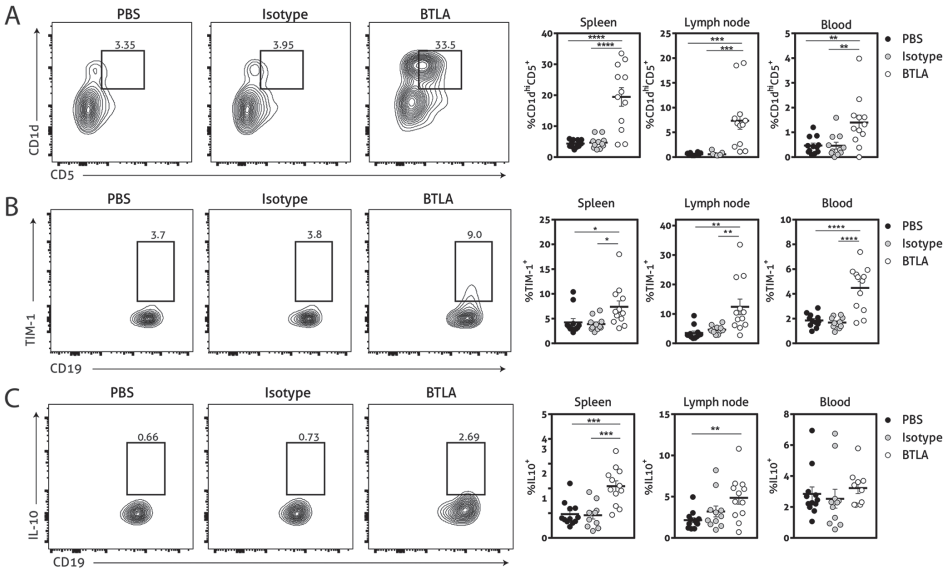


Figure 4. Activation of BTLA leads to increased regulatory B cells.
Female *Ldlr*$^{-/-}$ mice were treated twice a week with PBS, an isotype antibody or an agonistic BTLA antibody and fed a WTD for 6 weeks. Flow charts for splenocytes and quantifications of regulatory B cells are shown for **(A)** B10 cells (CD19$^+$CD1d^{hi}CD5$^+$), **(B)** TIM-1$^+$ B cells (CD19$^+$TIM-1$^+$) and **(C)** IL-10 secreting B cells (CD19$^+$IL-10$^+$). An ordinary one-way ANOVA test followed by a Holm-Sidak post hoc test was performed. Data are shown as mean ± SEM, n=11-12 (*p<0.05, ** p<0.01, ***p<0.001, **** p<0.0001).

on changes in DCs, as we did not find differences in total DCs or regulatory DCs (DEC-205$^+$CD8$^+$) between PBS-, isotype- or BTLA-treated mice (Online Figure X). To test if the altered CD4$^+$ T cell response after BTLA treatment was a direct effect of a different B cell pool, we cultured OT-II CD4$^+$ T cells with B cells from mice treated with PBS or the BTLA antibody. We found that after 72 hours of stimulation with OVA323 peptide, OT-II CD4$^+$ T cells cultured in the presence of BTLA-treated B cells, showed a marked increase in Tregs (Figure 5D). Moreover, CD4$^+$ T cells from co-cultures with BTLA-treated B cells showed a significant decrease in TNFα secretion, while IL-5 secretion was increased compared to T cells from co-cultures with PBS-treated B cells (Figure 5E). Finally, we show that the anti-inflammatory T cell response seen with BTLA agonism was not exclusively caused by changes seen in B cells, as BTLA treatment in B cell depleted mice also inhibits TNFα secretion by CD4$^+$ T cells (Online Figure XI).

Taken together, these data strongly indicate that the BTLA antibody reduced the peripheral activation of CD4$^+$ T cells, both indirectly by altering the APC function of B cells and by directly polarizing CD4$^+$ T cells, resulting in reduced lesional CD4$^+$ T cells.

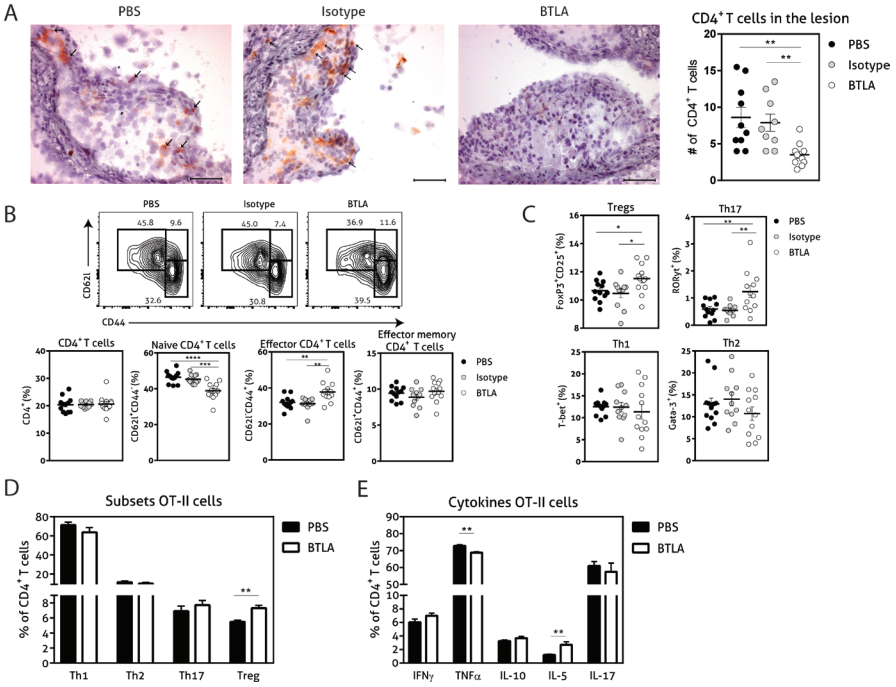


Figure 5. Activation of BTLA leads to decreased T cell infiltration and activation. Female *Ldlr*$^{-/-}$ mice were treated twice a week intraperitoneally with PBS, an isotype antibody or an agonistic BTLA antibody for 6 weeks while being fed a Western type diet. **(A)** Representative images of aortic root sections stained for CD4 (arrows) and hematoxyline and cell number quantification. Scale bars are 100 µm. **(B)** Flow charts and quantifications of total CD4$^+$ T cells, naive CD4$^+$ T cells (CD62l$^+$CD44$^-$), effector CD4$^+$ T cells (CD62l$^-$CD44$^+$) and effector memory CD4 T cells (CD62l$^+$CD44$^+$) in splenocytes. **(C)** Flow cytometry quantifications of regulatory T cells (FoxP3$^+$), Th17 (RORγt$^+$), Th1 (T-bet$^+$), and Th2 (Gata-3$^+$) cells in splenocytes. **(D)** B cells from mice treated with PBS or an agonistic BTLA antibody for 2 weeks were co-cultured with isolated CD4$^+$ T cells from OTII mice in the presence of OVA323 peptide (1 µg/ml). CD4$^+$ T cells were harvested after 72 hours and assessed with flow cytometry for regulatory T cells (FoxP3$^+$), Th17 (RORγt$^+$), Th1 (T-bet$^+$), and Th2 (Gata-3$^+$) cells and **(E)** for cytokine production. An ordinary one-way ANOVA test followed by a Holm-Sidak post hoc test was performed. Data are shown as mean ± SEM, n=9/12 (*p<0.05, ** p<0.01, ***p<0.001, ****p<.00001). Tregs; regulatory T cells, Th; T helper cell.

Activation of BTLA leads to increased stability in established lesions

Since most CVD patients that require medication already have well-established atherosclerosis, we also investigated the effects of the BTLA antibody on pre-existing lesions. Therefore, we fed *Ldlr*$^{-/-}$ mice a WTD for 10 weeks, after which we started the agonistic BTLA or isotype treatment for 6 weeks (Figure 6A). In line with our initial atherosclerosis study, we found a highly significant reduction in total B cells in all relevant organs (Figure 6B) caused by a strong decrease in follicular B cells (Figure 6C). In contrast, IL-10$^+$ B cells (Figure 6D) and Tregs were increased (Online

Figure XII). Although we did not find differences in lesion size between isotype- or BTLA-treated mice (Figure 6E), we did observe that lesions of mice treated with the BTLA antibody contained significantly more collagen than lesions of mice treated with the isotype control (Figure 6F). Furthermore, lesional macrophages, smooth muscle cell content and necrotic core area was determined (Online Figure XIII). This shows that BTLA stimulation increased the stability of already established lesions in *Ldlr*$^{-/-}$ mice.

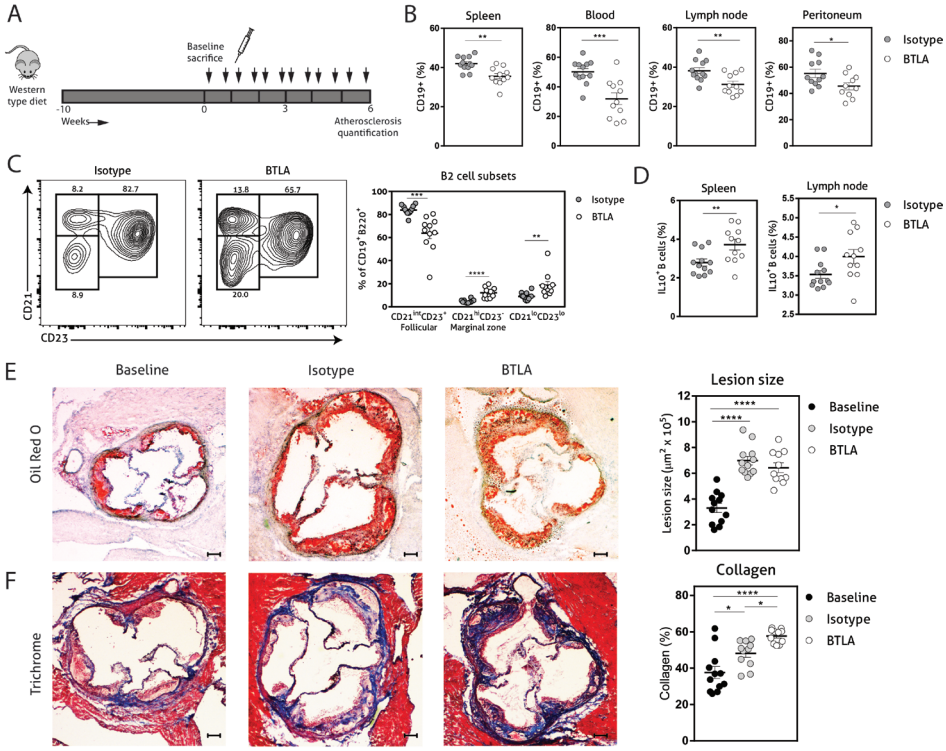


Figure 6. Activation of BTLA leads to increased stability in established lesions.
(A) Female *Ldlr*$^{-/-}$ mice were fed a WTD for 10 weeks after wich one group was sacrificed (baseline), while other mice were treated twice a week intraperitoneally with an isotype antibody or an agonistic BTLA antibody for 6 weeks. **(B)** Flow cytometry quantification of B cells (CD19^{+}) in different organs. **(C)** Flow charts of splenocytes and quantification of follicular (CD21^{int}CD23^{+}), marginal zone (CD-21^{hi}CD23^{-}) and newly formed (CD23^{lo}CD21^{lo}) B cells in the spleen. **(D)** Flow cytometry quantification of regulatory B cells (CD19^{+}IL-10^{+}) in spleen and lymph node. **(E)** Oil-Red-O and hematoxylin staining in aortic root sections and lesion size quantification. **(F)** Trichrome staining in aortic root sections and collagen quantification. Scale bars are 100 µm. A Student's t-test was performed for two groups or an ordinary one-way ANOVA test followed by a Holm-Sidak post hoc test for multiple groups. Data are shown as mean ± SEM n=11-12 (*p<0.05, ** p<0.01, ***p<0.001, **** p<0.0001).

Discussion

The discovery of immune checkpoint proteins has been a tremendous support in finding targets for immunomodulatory drugs [2]. For many of these co-receptors, therapeutic antibodies or small molecules are now being tested in experimental animal models or in clinical trials. For the treatment of atherosclerosis and CVD we still lack potent agents that target pathways other than cholesterol metabolism. There is an urgent need for such treatments, since the residual risk of CVD is still considerable even after effective cholesterol management [27]. Moreover, the recent CANTOS trial demonstrated that anti-inflammatory therapy can significantly reduce the risk of cardiovascular events [28]. In this study, we provide the first evidence that one of the newest additions to the co-receptor family, BTLA, is a very promising target for the treatment of atherosclerosis.

Up to date, most research in relation to BTLA has focused on T cells, while it is most abundantly expressed on B cells [3,6,29,30]. One apparent reason for this discrepancy could be that many inflammatory disorders are thought to be mainly T cell driven. However, with the recognition of novel B cell subsets, we gained significantly more insight into the actual contribution of B cells to inflammatory disorders. In atherosclerosis, we now know that B1 cells [19,31], marginal zone B cells [25] and regulatory B cells [32] can exert protective functions, contrary to follicular B cells that aggravate atherosclerosis [20,21]. Since follicular B cells are in the majority, complete B2 cell depletion has resulted in attenuated atherosclerosis [20,21]. Yet, these treatments deplete the complete B2 cell population which includes atheroprotective Bregs and marginal zone B cells [20,21,33]. In a side-to-side comparison study, we show that whereas anti-CD20 treatment depletes all B cells, BTLA specifically targets follicular B2 cells and can directly inhibit pro-inflammatory $CD4^+$ T cells. Secondly, the humoral immunity is compromised with complete B2 cell depletion, illustrated by reductions in both total and antigen-specific IgG and IgM antibodies [20,21]. In this study, we now describe that an agonistic antibody for BTLA specifically reduced, but not ablated, the atherogenic follicular B cells, while the atheroprotective B cell subsets were increased. Although B cell depleting antibodies are already in clinical use for other autoimmune diseases such as rheumatoid arthritis[34], we believe that BTLA stimulation might be superior to total B cell depletion in CVD.

Additionally, we have found that the humoral immunity was not affected since both total and antigen-specific IgG1, IgG2 and IgM levels remained the same. This is in line with others that showed that blockade of the HVEM/BTLA pathway also did not

alter the humoral effect following transplantation [35]. This highlights the potential benefits of using an agonistic BTLA antibody for the treatment of atherosclerosis.

Besides reducing atherogenic follicular B2 cells, we found that BTLA activation resulted in a splenic B cell population enriched in atheroprotective marginal zone B cells and Bregs. Bregs have shown great protective potential in autoimmune disorders such as collagen-induced arthritis and experimental autoimmune encephalomyelitis, primarily through their production of IL-10 [22]. Studies investigating the role of Bregs in atherosclerosis show conflicting results [18,32,36]. However, recently we found a very strong inverse correlation between atherosclerosis severity and the frequency of IL-10$^+$ B cells, and adoptive transfer of IL-10$^+$ B cells strongly reduced circulating leukocyte numbers and inflammatory monocytes in *Ldlr*$^{-/-}$ mice[37] Moreover, blockade of TIM-1, another immune checkpoint protein strongly associated with increased IL-10 producing B cells [24], leads to aggravated atherosclerosis, potentially via the blockade of TIM-1$^+$ B cells [38]. Since BTLA agonism strongly increased B10 cells and TIM-1$^+$ B cells, we believe this contributed to the reduction of atherosclerosis.

In the last years, it has been increasingly recognized that B cells have an important cellular function independent of antibody production. We found that the altered B cell population led to strongly decreased T cell activation. In addition, we showed that in both studies BTLA activation resulted in increased Tregs. During many autoimmune disorders, including atherosclerosis, Tregs become dysfunctional and are unable to curb disease [26]. Restoration of this function or expansion of Tregs has been protective in atherosclerosis [26]. Interestingly, it has previously been demonstrated that in experimental autoimmune encephalomyelitis, Bregs are able to recover the inhibitory activity of Tregs in a BTLA-dependent manner [39]. Enhanced inhibitory activity of Tregs, could also explain the decrease in T cell activation found in this study. Furthermore, we show that the BTLA antibody also directly inhibits pro-inflammatory T cells under hypercholesterolemic conditions. Overall, the expansion of Bregs and Tregs could have greatly contributed to the atheroprotective effects found with BTLA activation.

Nowadays, tremendous efforts are undertaken in the clinic to identify and treat vulnerable lesions that are prone to rupture [40]. By stimulating the BTLA pathway we were able to both reduce lesion size in an initiation study and also stabilize lesions during progression of atherosclerosis. The latter underscores that modulating the BTLA pathway presents a very promising option for clinical use. In addition, we found that in CVD patients almost 90% of all circulating B cells still express high

levels of BTLA. Despite our limited sample number, this suggests that BTLA is also an interesting and accessible target in CVD patients.

In summary, treatment with an agonistic BTLA antibody prevents atherosclerosis and stabilizes already established lesions by favorably shifting the balance between atherogenic follicular B cells and atheroprotective B cells and directing $CD4^+$ T cells towards Tregs. Our data strongly indicate that BTLA activation may be considered for the treatment of atherosclerosis.

Acknowledgements

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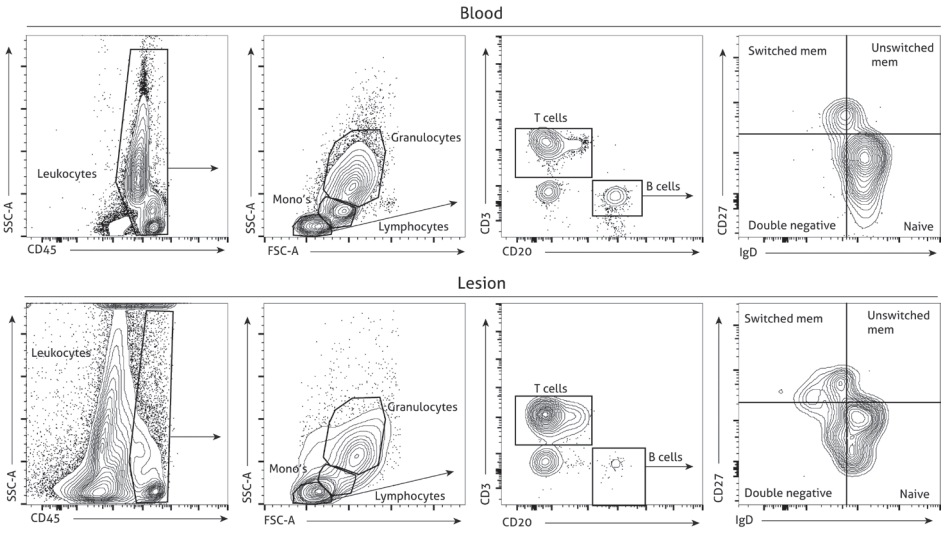
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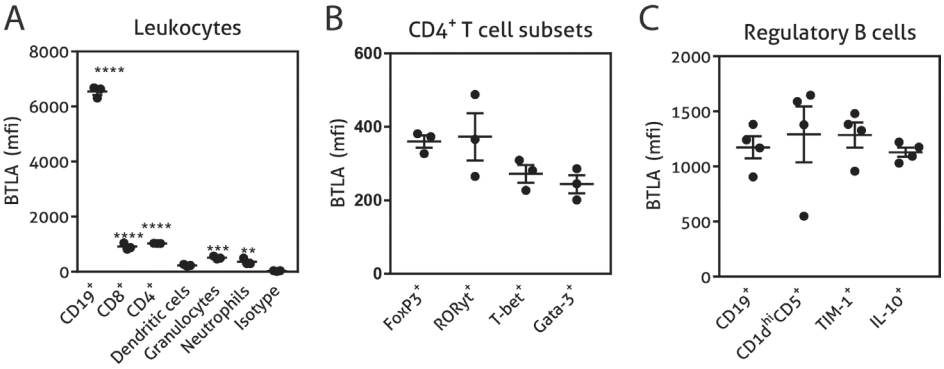
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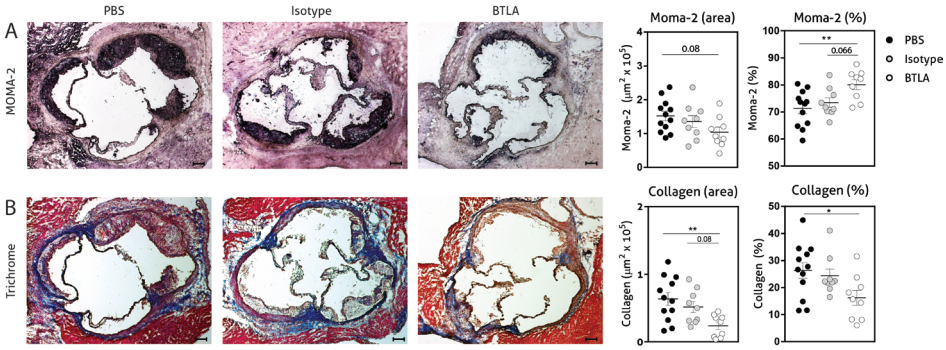
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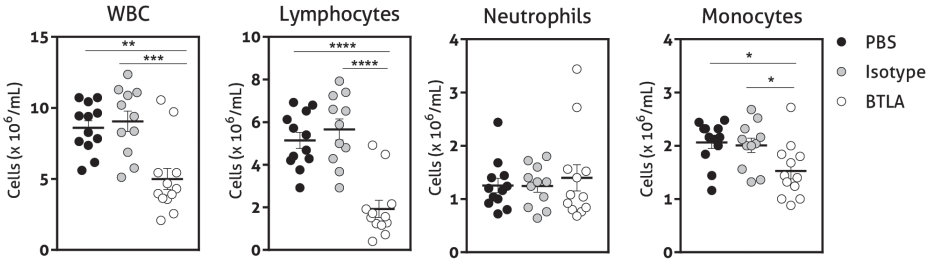
Online Figure I. **Gating strategy leukocyte population blood and lesion CVD patients.**
Flow cytometry was applied on blood and lesions from CVD patients to identify BTLA expression on major leukocyte populations. Cells were analyzed for a live/dead marker after which the indicated gating strategies were applied. B cells were also assessed for CD19 expression before subtypes were identified.



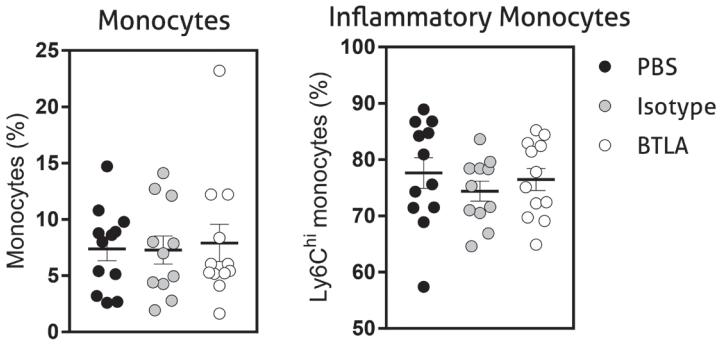
Online Figure II. **BTLA expression of CD4$^+$ T cell subsets in *Ldlr*$^{-/-}$ mice.**
(A) Quantification of BTLA expression in leukocyte populations; dendritic cells (CD19$^-$SSC^{lo}CD11c$^+$CD11b$^+$), granulocytes (SSC^{hi}CD11b$^+$), neutrophils (CD11b$^+$Ly-6G$^+$). **(B)** Quantification of BTLA expression in effector CD4$^+$ T cell subsets; Th1 (T-bet$^+$), Th2 (Gata-3$^+$), Th17 (RORγt$^+$) and Tregs (FoxP3$^+$). **(C)** Quantification of BTLA expression in regulatory B cell subsets. Data are shown as mean ± SEM, n=3. Tregs; regulatory T cells, Th; T helper cell. Data are shown as mean ± SEM. An ordinary one-way ANOVA test followed by a Holm-Sidak post hoc test was performed. (** p<0.01, ***p<0.001, **** p<0.0001 vs. isotype).



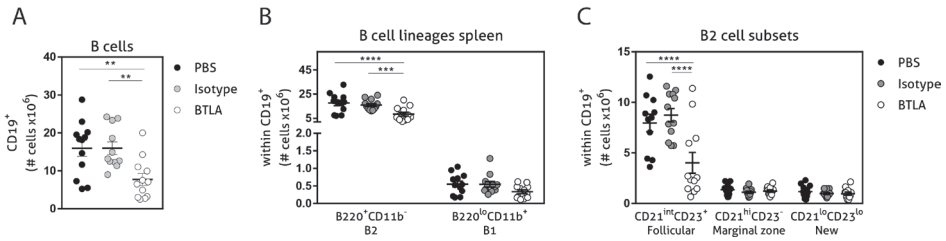
Online Figure III. **Activation of BTLA leads to an early lesion phenotype.**
Female *Ldlr*$^{-/-}$ mice were treated twice a week intraperitoneally with PBS, an isotype antibody or an agonistic BTLA antibody for 6 weeks while being fed a Western type diet. **(A)** MOMA-2 staining in aortic root sections with total and relative macrophage area quantification. **(B)** Trichrome staining in aortic root sections with total and relative collagen area quantification. Scale bars are 100 μm. Data are shown as mean ± SEM. An ordinary one-way ANOVA test followed by a Holm-Sidak post hoc test was performed. (*p<0.05, ** p<0.01).



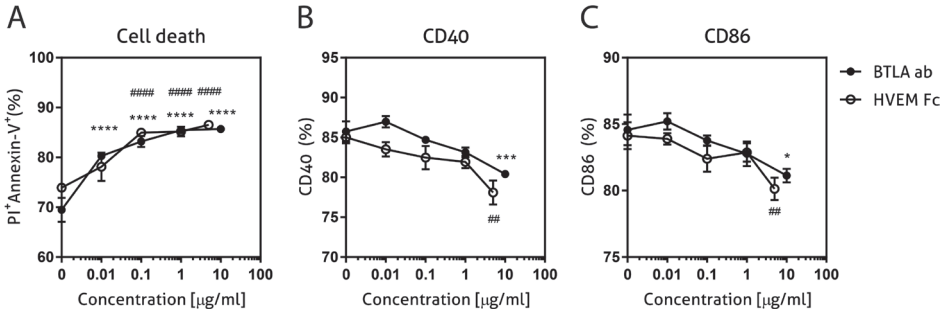
Online Figure IV. **Activation of BTLA leads to a strong reduction in circulation lymphocytes.**
Female *Ldlr*$^{-/-}$ mice were treated twice a week intraperitoneally with PBS, an isotype antibody or an agonistic BTLA antibody for 6 weeks while being fed a Western type diet. After sacrifice, circulating levels of total white blood cells (WBC), lymphocytes, neutrophils or monocytes were measured in full blood using a hematology analyzer. Data are shown as mean ± SEM. An ordinary one-way ANOVA test followed by a Holm-Sidak post hoc test was performed. (*p<0.05, ** p<0.01, ***p<0.001, ****p<0.0001).



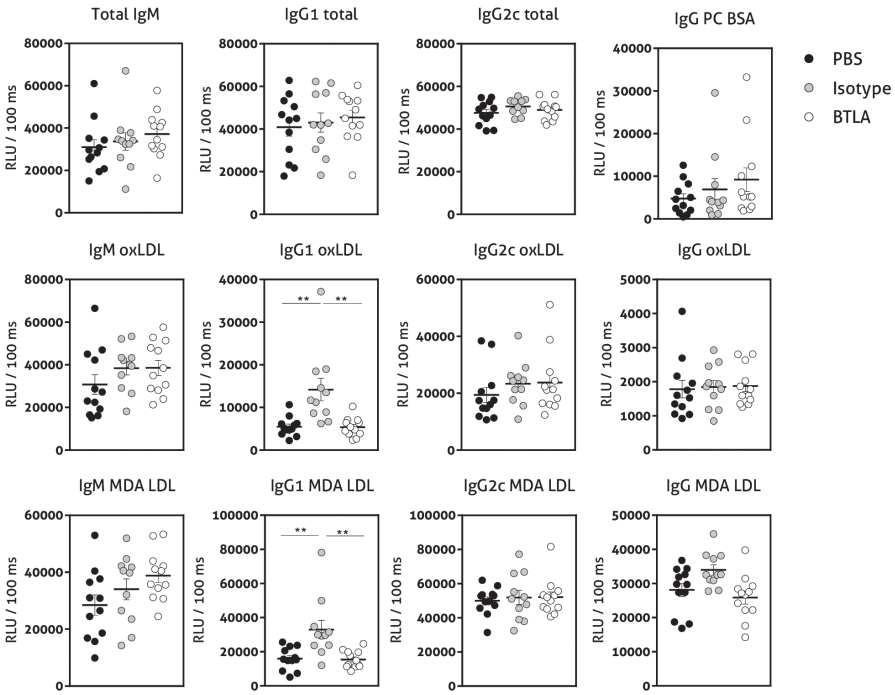
Online Figure V. **Activation of BTLA does not lead to changes in monocytes.** Female *Ldlr*$^{-/-}$ mice were treated twice a week intraperitoneally with PBS, an isotype antibody or an agonistic BTLA antibody for 6 weeks while being fed a Western type diet. Cirulating monocytes (B220^{-}NK1.1^{-}CD11b^{+}SSC^{int}Ly-6G^{lo}CD115^{+}) and inflammatory monocytes (B220^{-}NK1.1^{-}CD11b^{+}SSC^{int}Ly-6G^{lo}CD115^{+}Ly-6C^{hi}) were measured with flow cytometry. Data are shown as mean ± SEM.



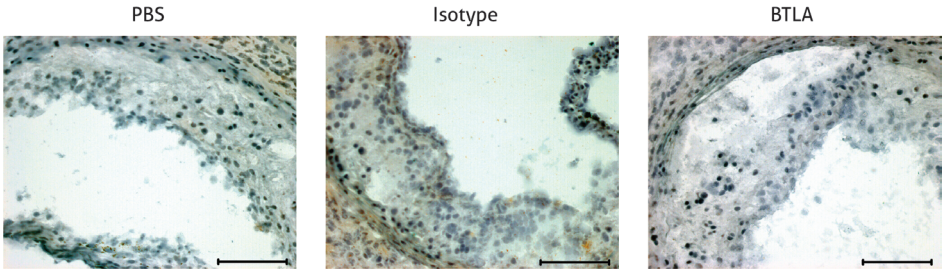
Online Figure VI. **Activation of BTLA leads to a strong reduction in abolute numbers of follicular B2 cells in the spleen.** Female *Ldlr*$^{-/-}$ mice were treated twice a week intraperitoneally with PBS, an isotype antibody or an agonistic BTLA antibody for 6 weeks while being fed a Western type diet. **(A)** Quantification of absolute number of B cells (CD19^{+}) in th spleen. **(B)** Quantification of absolute number of B1 cells (CD19^{+}CD11b^{+}B220^{lo}) and B2 cells (CD19^{+}CD11b^{-}B220^{+}) in the spleen. **(C)** Quantification of absolute number of follicular (CD21^{int}CD23^{+}), marginal zone (CD21^{hi}CD23^{-}) and newly formed (CD23^{lo}CD21^{lo}) B cells in the spleen. Data are shown as mean ± SEM. An ordinary one-way ANOVA test followed by a Holm-Sidak post hoc test was performed. (*p<0.05, ** p<0.01).



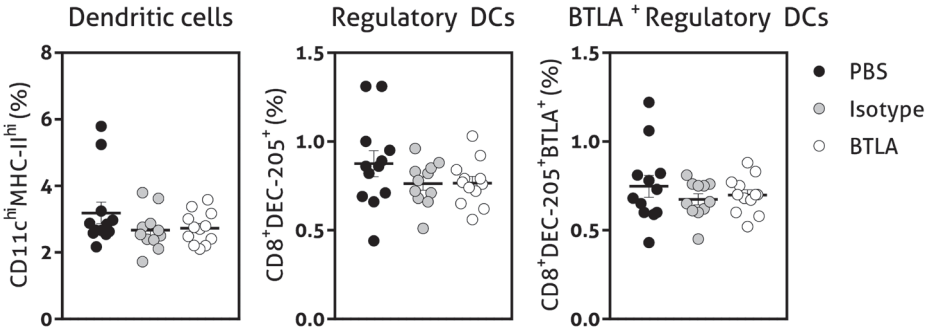
Online Figure VII. **Activation of BTLA leads increased cell death and reduced activation in B cells.** Splenic B cells were isolated from female *Ldlr*$^{-/-}$ mice with CD19^{+} microbeads and stimulated with different concentrations of an agonistic BTLA antibody or recombinant murine HVEM Fc for 24h. **(A)** Cell death was measured with propidium iodide and Annexin-V staining. B cell activation was measured using CD40 **(B)** or CD86 **(C)**. Data are shown as mean ± SEM. An ordinary one-way ANOVA test followed by a Holm-Sidak post hoc test was performed. (*p<0.05, ***p<0.001, ****p<0.0001 BTLA ab vs 0 and ##p<0.01, ####p<0.0001 HVEM Fc vs 0).



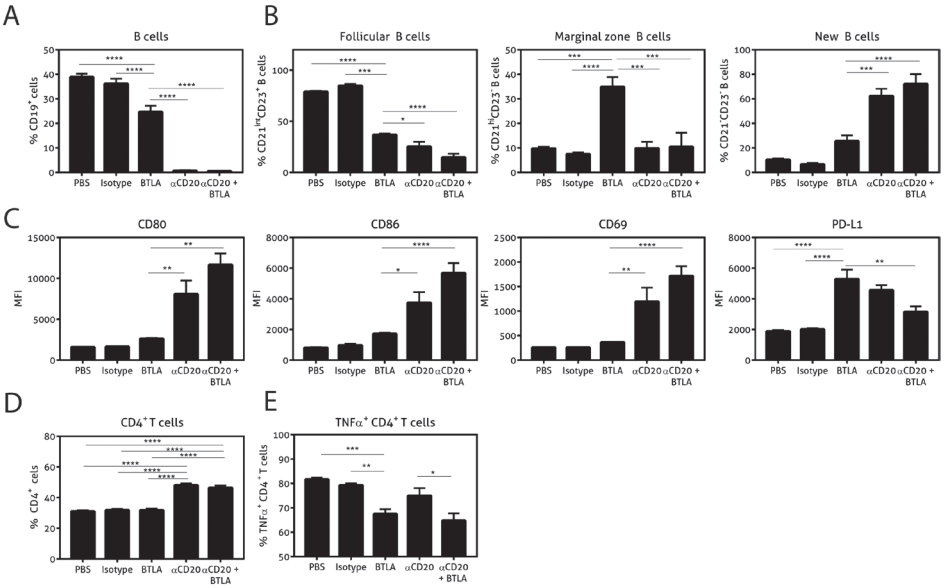
Online Figure VIII. **BTLA activation does not affect the humoral immune response.** Female *Ldlr*$^{-/-}$ mice were treated twice a week intraperitoneally with PBS, an isotype antibody or an agonistic BTLA antibody for 6 weeks while being fed a Western type diet. At euthanasia, serum of mice was collected in which total and antigen-specific antibodies for atherosclerosis-related antigens were determined using ELISA. Data are shown as mean ± SEM. An ordinary one-way ANOVA test followed by a Holm-Sidak post hoc test was performed. (** p<0.01). oxLDL, oxidized low-density lipoprotein; MDA-LDL, malondialdehyde-modified low-density lipoprotein; BSA, bovine serum albumin; RLU relative light units; PC, phosphatidylcholine.



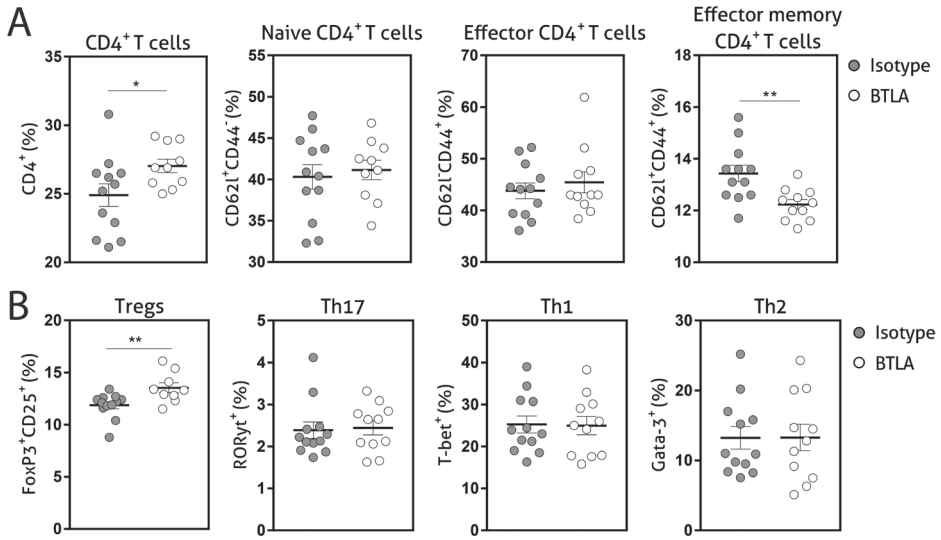
Online Figure IX. **Absence of B220$^+$ cells in the aortic root.** Female *Ldlr*$^{-/-}$ mice were treated twice a week intraperitoneally with PBS, an isotype antibody or an agonistic BTLA antibody for 6 weeks while being fed a Western type diet. Representative images of aortic root sections stained for B220 (brown) and hematoxylin (blue) are shown. No B220$^+$ cells were observed. Scale bars are 100 μm.



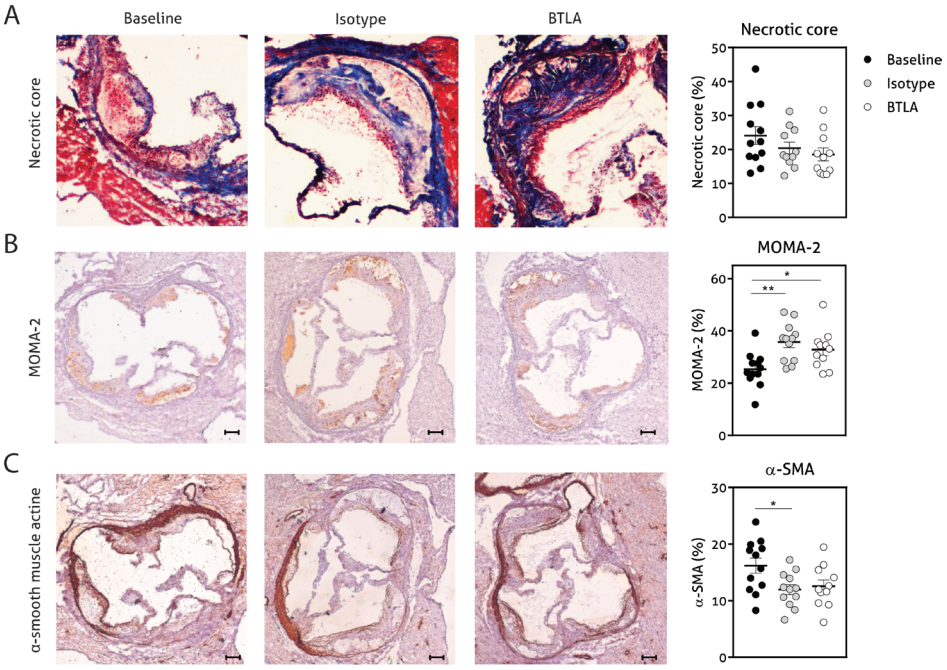
Online Figure X. **Activation of BTLA does not lead to changes in dendritic cells.** Female *Ldlr*$^{-/-}$ mice were treated twice a week intraperitoneally with PBS, an isotype antibody or an agonistic BTLA antibody for 6 weeks while being fed a Western type diet. Dendritic cells (CD19$^-$CD11c$^+$MHC-II^{hi}), regulatory dendritic cels (CD19$^-$CD11c$^+$MHC-II^{hi}CD8$^+$DEC-205$^+$) and BTLA$^+$ regulatory dendritic cells were measured with flow cytometry. Data are shown as mean ± SEM.



Online Figure XI. Female *Ldlr*$^{-/-}$ mice (n=5-6/group) were fed a WTD for 2 weeks while receiving intraperitoneal injections with PBS, an isotype antibody, an agonistic BTLA antibody, anti-CD20 or anti-CD20+BTLA. **(A)** Flow cytometry quantifications of total CD19^{+} T cells. **(B)** Flow cytometry quantifications of follicular B cells (CD21^{int}CD23^{+}), marginal zone B cells (CD21^{hi}CD23^{-}) and new B cells (CD21^{-}CD23^{-}) within the CD19^{+} population. **(C)** Flow cytometry quantifications of co-receptor expression on CD19^{+} B cells. **(D)** Flow cytometry quantification of CD4^{+} T cells and (E) of TNFα^{+} CD4^{+} T cells. Data are shown as mean ± SEM. A One-way ANOVA was performed (*p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001).



Online Figure XII. **BTLA activation leads to changes in CD4 T cells.** Female *Ldlr*$^{-/-}$ mice were fed a WTD for 10 weeks after wich one group was sacrificed (baseline) and other mice were treated twice a week intraperitoneally with an isotype antibody or an agonistic BTLA antibody for 6 weeks **(A)** Flow cytometry quantifications of total CD4$^+$ T cells, naive CD4$^+$ T cells (CD62l$^+$CD44$^-$), effector CD4$^+$ T cells (CD62l$^-$CD44$^+$) and effector memory CD4 T cells (CD62l$^+$CD44$^+$) in splenocytes. **(B)** Flow cytometry quantifications of regulatory T cells (FoxP3$^+$), Th17 (RORγt$^+$), Th1 (T-bet$^+$), and Th2 (Gata-3$^+$) cells in splenocytes. (C) Data are shown as mean ± SEM. A Student's t-test was performed (*p<0.05, ** p<0.01). Tregs; regulatory T cells, Th; T helper cell.



Online Figure XIII. **Activation of BTLA promotes plaque stabilization.** Female *Ldlr*$^{-/-}$ mice were fed a WTD for 10 weeks after wich one group was sacrificed (baseline) and other mice were treated twice a week intraperitoneally with an isotype antibody or an agonistic BTLA antibody for 6 weeks. **(A)** Representative pictures and quantification of necrotic core analysis from Masson's Trichrome stained sections. **(B)** Representative pictures of MOMA-2 staining in aortic root sections with macrophage quantification. **(C)** Representative pictures of alpha-smooth muscle staining in aortic root sections with smooth muscle cell quantification. Scale bars are 100 µm. Data are shown as mean ± SEM. A One-way ANOVA was performed (*p<0.05, ** p<0.01).

Table 1

Atherosclerosis initiation study (acquired on FACS canto II)			
Treg/Th17			
Antigen	**Label**	**Clone**	**Supplier**
Dead/Live	APC-Cy7	n/a	Thermo Fisher
CD4	PerCP	RM4-5	BD Biosciences
CD25	APC	PC61.5	Thermo Fisher
CD44	FITC	IM7	Thermo Fisher
FoxP3	Pacific Blue	FJK-16s	Thermo Fisher
RORyt	PE	AFKJS-9	Thermo Fisher
Th1/Th2			
Dead/Live	APC-Cy7	n/a	Thermo Fisher
CD8	Alexa fluor 700	53-6.7	Biolegend
CD4	PerCP	RM4-5	BD Biosciences
CD62l	FITC	MEL-14	Thermo Fisher
CD44	Pe-Cy7	IM7	Thermo Fisher
T-bet	Alexa fluor 647	4B10	Thermo Fisher
Gata-3	PE	TWAJ	Thermo Fisher
B1/B2 cells			
Dead/Live	APC-Cy7	n/a	Thermo Fisher
CD19	PeCy7	1D3	Thermo Fisher
B220	FITC	RA3-6B2	Thermo Fisher
CD5	BV421	53-7.3	BD Biosciences
CD11b	APC	M1/70	Thermo Fisher
B2 cell subsets			
Dead/Live	APC-Cy7	n/a	Thermo Fisher
CD19	PeCy7	1D3	Thermo Fisher
CD21	BV421	7G6	Thermo Fisher
CD23	PE	B3B4	Thermo Fisher
CD93	PerCP-Cy 5.5	AA4.1	Thermo Fisher
IgD	APC	11-26	Thermo Fisher
IgM	FITC	II/41	Thermo Fisher
Regulatory B cells			
Dead/Live	APC-Cy7	n/a	Thermo Fisher
CD19	PeCy7	1D3	Thermo Fisher
CD5	BV421	53-7.3	BD Biosciences
CD1d	PerCP-Cy 5.5	1B1	Thermo Fisher
TIM-1	PE	RMT1-4	Biolegend
CD9	FITC	KMC8	Thermo Fisher
IL-10	APC	JES5-16E3	eBioscience

BTLA expression in human tissue			
B cell subsets			
Antigen	Label	Clone	Supplier
Dead/Live	APC-efluor 780	n/a	Thermo Fisher
CD19	PercP-Cy5.5	HIB19	Thermo Fisher
CD20	FITC	2H7	Thermo Fisher
CD27	Brilliant Violet 605	O323	Biolegend
CD45	APC	5B1	Miltenyi Biotec
BTLA	Pe-Dazzle	MIH-26	Biolegend
CD3	eFluor 450	OKT3	Thermo Fisher
IgD	Alexa fluor 700	IA6-2	Biolegend

Atherosclerosis progression study (acquired on Cytoflex S)			
B cell subsets			
Antigen	Label	Clone	Supplier
Dead/Live	APC-efluor 780	n/a	Thermo Fisher
CD93	PE-Cy7	AA4.1	Thermo Fisher
CD19	BV605	6D5	Biolegend
CD43	APC	S11	Biolegend
CD5	FITC	53-7.3	Thermo Fisher
CD21	BV421	7E9	Biolegend
CD23	PE	B3B4	Thermo Fisher
B220	PerCP-Cy5.5	RA3-6B2	Thermo Fisher
T cell subsets			
Dead/Live	APC-efluor 780	n/a	Thermo Fisher
CD4	PerCP	RM4-5	BD Biosciences
CD8a	Alexa fluor 700	53-6.7	Biolegend
CD44	BV510	IM7	Biolegend
CD62L	FITC	MEL-14	Thermo Fisher
CD25	APC	PC61.5	Thermo Fisher
FoxP3	Pacific blue	FJK-16s	Thermo Fisher
T-bet	PE-Cy7	eBio4B10	Thermo Fisher
RORyt	BV650	Q31-378	BD Biosciences
Gata-3	PE	TWAJ	Thermo Fisher
Regulatory B cells			
Dead/Live	APC-Cy7	n/a	Thermo Fisher
CD19	BV605	6D5	Biolegend
CD5	BV421	53-7.3	BD Biosciences
CD1d	PerCP-Cy 5.5	1B1	Thermo Fisher
TIM-1	PE	RMT1-4	Biolegend
IL-10	APC	JES5-16E3	eBioscience

BTLA expresion in LDLRKO mice (acquired on Cytoflex S)			
B cell subsets			
Antigen	Label	Clone	Supplier
Dead/Live	APC-efluor 780	n/a	Thermo Fisher
CD93	PE-Cy7	AA4.1	Thermo Fisher
CD19	BV605	6D5	Biolegend
BTLA	APC	8F4	Biolegend
CD5	FITC	53-7.3	Thermo Fisher
CD21	BV421	7E9	Biolegend
CD23	PE	B3B4	Thermo Fisher
B220	PerCP-Cy5.5	RA3-6B2	Thermo Fisher
T cell subsets			
Dead/Live	APC-efluor 780	n/a	Thermo Fisher
CD4	PerCP	RM4-5	BD Biosciences
CD8a	Alexa fluor 700	53-6.7	Biolegend
CD44	BV510	IM7	Biolegend
CD62L	FITC	MEL-14	Thermo Fisher
BTLA	APC	8F4	Thermo Fisher
FoxP3	Pacific blue	FJK-16s	Thermo Fisher
T-bet	PE-Cy7	eBio4B10	Thermo Fisher
RORyt	BV650	Q31-378	BD Biosciences
Gata-3	PE	TWAJ	Thermo Fisher
T cell subsets			
Dead/Live	APC-efluor 780	n/a	Thermo Fisher
CD8a	APC-efluor 700	53-6.7	Biolegend
CD19	PE-Cy7	1D3	Thermo Fisher
CD4	PerCP	RM4-5	BD Biosciences
CD11c	FITC	N418	Thermo Fisher
CD11b	BV421	M1/70	Thermo Fisher
LY-6G	PE	1A8	BD Biosciences
F4/80	Brilliant Violet 650	T45-2342	BD Biosciences
BTLA	APC	8F4	Thermo Fisher

General Discussion

Summary

Atherosclerosis is the main underlying cause for cardiovascular disease (CVD). Atherosclerosis is characterized by the build-up of cholesterol in the artery wall. For this reason, cholesterol and lipid metabolism have been studied extensively in the pathogenesis of atherosclerosis during the 20th century. This has led to advancements in treatment options including the use of statins that block the rate-limiting enzyme in the cholesterol synthesis. Statins significantly lower cholesterol levels and are able to reduce cardiovascular relative risk by 25-30%. More novel approaches to lower cholesterol levels include the use of PCSK9 inhibitors, which have also shown to significantly reduce the relative risk of cardiovascular events1. Despite these lipid-lowering drugs, CVD remains a central health care problem in the Western world, with large numbers of patients and CVD-related deaths2. We nowadays know that besides lipid metabolism, the immune system is a major contributor during all stages of atherosclerosis.

The first paper that suggested an active immune-inflammatory pathway during atherosclerosis development appeared in 1985[3]. This paper demonstrated that a large number of cells present in carotid artery plaques expressed MHCII molecules, which suggested that antigen presentation to and activation of $CD4^+$ T cells could be part of the atherosclerosis pathophysiology. Interestingly, the same paper also demonstrated that 30% of all cells present in the lesion expressed Fc-receptors, indicating that the humoral immunity might also be an important process in atherosclerosis[3]. This latter fact, however, did not receive the same attention as the first one and we can see in Figure 1 that in the years after this initial publication, the number of studies investigating $CD4^+$ T cells and atherosclerosis grew exponentially, while the number of publications about B cells and atherosclerosis did not increase that much. A potential reason for this discrepancy could be that for most antigens, antibody production by B cells requires an effective $CD4^+$ T cell response. Thus research into $CD4^+$ T cells indirectly also investigated the humoral immunity. Nowadays, we know that many antigens can elicit a T cell-independent B cell response. Additionally, more and more antibody-independent functions of B cells have been discovered in the last decade, which has resulted in a renewed interest in the role of B cells in atherosclerosis (Fig. 1).

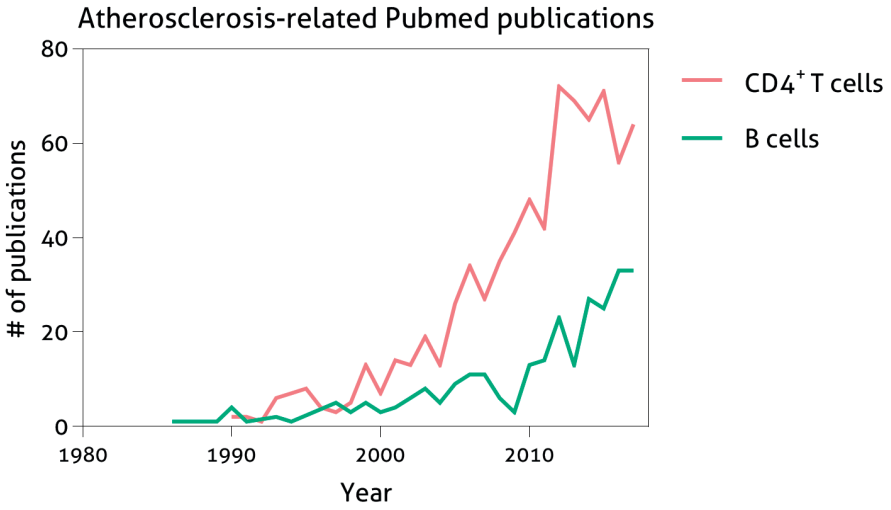


Figure 1. Number of indexed articles in Pubmed. A basic Pubmed search for "CD4" or "B cells" together with "atherosclerosis" was performed (24/10/18). The number of resulting publications are plotted (y-axis) versus the year of publication (x-axis).

This thesis

in this thesis, we aimed to explore and modulate the B cell response during atherosclerosis. In chapter 2 we provide an overview of the current knowledge regarding the role of B cells in atherosclerosis. In general, B cells can be divided in two main lineages; B1 and B2 cells (Fig. 2). These B cells are different in origin and function which is also resembled by their differential effects in atherosclerosis. B1 cells form a minor B cell population and thought to be atheroprotective, while B2 cells comprise the majority of B cells and have a proatherogenic role. The distinction between B1 and B2 cells is, however, a simplified representation and both B1 and B2 cells can be further divided into B cell subsets. For example, it has recently been shown that marginal zone B2 cells have a protective role during atherosclerosis[4]. On the other hand, the majority of B2 cells are of the follicular phenotype which are known to aggravate atherosclerosis[5]. In chapter 6 we targeted these follicular B cells with an agonistic BTLA antibody. Besides these classical B cell subsets, novel B cell subsets that are characterized by specific functional properties have now been identified. For instance, in chapter 3 we examined the role of IL-10-producing B cells. Similarly, in chapter 4 we used TIM-1$^{\Delta mucin}$ mice that show a clear impairment in IL-10 secretion by B cells. Finally, in chapter 5 we focused on B cells that express PD-L1 through which they can inhibit T follicular helper (T_{FH}) cells. In these chapters

we used two main methods to modulate the B cell response; (1) through adoptive transfer of B cell subsets and (2) by modulation of co-receptors.

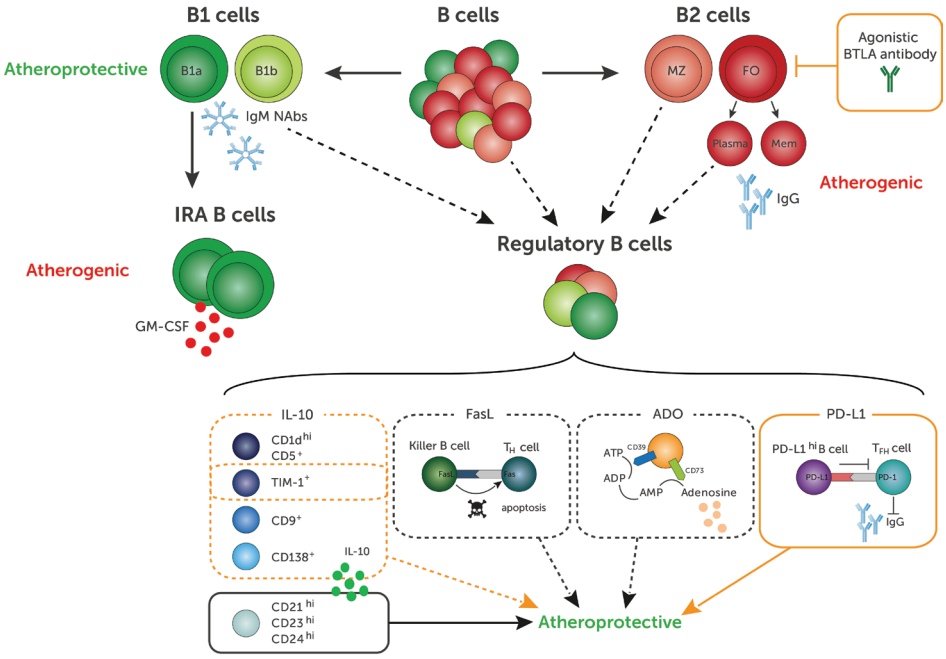


Figure 2. Overview of the main B cell subsets and their known and putative effects on atherosclerosis. Orange highlighted sections indicate the investigated B cell subsets in this thesis.

Cellular therapy by B cell adoptive transfers

The main rationale of anti-inflammatory therapy for atherosclerosis is to limit the local and systemic immune response. We hypothesized that cellular therapy with regulatory B cells would lead to immune cell inhibition and reduced atherosclerosis. Regulatory B cells form a family of different B cell subsets that are able to limit the immune response.

In chapter 3 we focused on B cells that produce the anti-inflammatory cytokine IL-10. These are the most extensively studied regulatory B cells in autoimmune disorders[6], but the role of IL-10$^+$ B cells in atherosclerosis was still controversial[7–11]. For this reason, we first set out to examine the dynamics of IL-10$^+$ B cells during atherosclerosis development. This showed that during disease progression, the number of IL-10$^+$ B cells gradually decreased. We also found a strong inverse correlation between IL-10$^+$ B cells and lesion size. We thus reasoned that adoptive transfer of IL-10$^+$ B cells would represent a beneficial treatment of atherosclerosis. The main

hurdle however with IL-10$^+$ B cells is that they are a relatively rare population and they cannot be flow-sorted with general procedures. Earlier work in other autoimmune disorders mainly resolved these problems by using specific B cell subsets in which IL-10$^+$ B cells are enriched (e.g. CD1d^{hi}CD5$^+$ B cells)[7]. Although enriched, the majority of these B cells are still IL-10$^-$ B cells. Therefore, we opted for a different strategy in which we stimulated *ex vivo* cultured B cells with anti-CD40 and subsequently used a specialized isolation kit to isolate pure IL-10$^+$ and IL-10$^-$ B cells. After adoptive transfer of IL-10$^+$ B cells we observed that they indeed reduced the inflammation as measured by circulating leukocytes and CD4$^+$ T cell activation, while adoptive transfer of IL-10$^-$ B cells did not result in any noticeable immune response limitation. Moreover, we found a significant increase in IL-10$^+$ CD4$^+$ T cells when mice were treated with IL-10$^+$ B cells. Unexpectedly, the immune regulation by IL-10$^+$ B cells did not result in reduced lesion formation, while IL-10$^-$ B cells strongly exacerbated atherosclerosis. To explain the lack of effect on lesion size by adoptive transfer of IL-10$^+$ B cells, we assessed other factors that could have influenced the development of atherosclerosis. This revealed that adoptive transfer of both IL-10$^-$ and IL-10$^+$ B cells resulted in hepatic steatosis. Both treatments increased the hepatic and circulating lipid levels, which are known to have very strong correlations with lesion size. Interestingly, this correlation was not present in mice treated with IL-10$^+$ B cells, suggesting that the anti-inflammatory effects had negated the lipid effects. In an effort to explain the increased lipid levels after B cell adoptive transfer, we investigated the expression of LIGHT and lymphotoxin-alpha on anti-CD40 stimulated B cells. We observed a very marked increase in both molecules, which have previously been shown to increase cholesterol levels. This could be the reason that both B cell treatments resulted in hepatic steatosis. Nonetheless, this study showed that IL-10$^+$ B cells were able to regulate the immune response during atherosclerosis development. For this reason, we believe that there is still ample evidence that other strategies which increase IL-10$^+$ B cells without affecting lipid metabolism, could ameliorate atherosclerosis.

In chapter 5, we performed another B cell adoptive transfer in which we specifically investigated B cells that express high levels of PD-L1, since it was demonstrated that these cells are able to inhibit CD4$^+$ T follicular helper (T_{FH}) cells[12]. Earlier studies already demonstrated a proatherogenic role of T_{FH} cells but did not report a therapeutic strategy to inhibit these cells and ameliorate atherosclerosis[4,13,14]. We first investigated the ratio of PD-L1^{hi} B cells and T_{FH} cells in young ApoE$^{-/-}$ mice with minimal lesions and in old atherosclerotic ApoE$^{-/-}$ mice with established lesions. We observed that the ratio was significantly increased, with relatively more T_{FH} cells than PD-L1^{hi} B cells in older ApoE$^{-/-}$ mice. Hence, we hypothesized that adoptive transfer

of PD-L1^{hi} B cells could be able to reduce atherosclerosis. Our first approach was to flow-sort PD-L1^{hi} B cells and adoptively transfer these to ApoE$^{-/-}$ mice. However, this did not result in significant T_{FH} restriction. We subsequently tried to optimize an *ex vivo* culture protocol to generate a large and pure population of PD-L1 expressing B cells. We showed that 24-hour stimulation of B cells with IFNγ resulted in very strong upregulation of PD-L1 on almost all B cells. Functionally, these IFNγ-B cells were able to inhibit T_{FH} cells both *in vitro* and *in vivo*. We thus adoptively transferred IFNγ-B cells to ApoE$^{-/-}$ mice in which we initiated lesion formation by placement of a perivascular collar. This led to an increase in PD-L1^{hi} germinal center B cells, plasmablast inhibition, increased regulatory T cells and a concomitant reduction in total lesion volume. This indicates that *ex vivo* stimulation of B cells with IFNγ, leads to a regulatory phenotype which is able to reduce atherosclerosis.

Taken together, these two chapters have shown the potential of cellular B cell therapy for the treatment of atherosclerosis. It is of particular interest that splenic B cells are mostly comprised of follicular B cells, which are known to be atherogenic[5]. Here, we have shown two *ex vivo* methods to generate and isolate protective B cell subsets from this general pool of atherogenic splenic B cells. The protective effects of our anti-CD40 generated IL-10^{+} B cells were, however, negated by their bidirectional effect which also induced increased cholesterol levels. The adoptive transfer of IFNγ-B cells or freshly isolated B cells, which were used as a control treatment in chapter 5, did not influence lipid metabolism. This further supports the notion that the increased cholesterol levels we observed in chapter 3 were due to our *ex vivo* protocol of anti-CD40 stimulation.

Although we have used a cellular adoptive transfer in chapter 5, PD-L1 is a well-known co-receptor and essentially this chapter could also have been categorized as modulation of co-receptors, which will be described below.

Modulation of co-receptors

A second approach through which we influenced the B cell response is by co-receptor modulation. Co-receptors are molecules which are mainly expressed by antigen-presenting cells and are key proteins in the immune response[15]. Co-receptors or immune-checkpoint proteins provide additional signals for a B or T cell which has recognized antigen. These signals can either be inhibiting or stimulating, resulting in a diminished or enhanced immune response respectively. Given their central role in the immune response, co-receptors are frequently targeted in auto-immune disorders. In atherosclerosis, stimulating coreceptors generally aggravate atherosclerosis, while inhibiting coreceptors are frequently atheroprotective[15]. In this thesis, we

have investigated the effects of TIM-1 deficiency in chapter 4 and of an agonistic antibody for BTLA in chapter 6.

T-cell immunoglobulin and mucin domain 1 (TIM-1) is part of a larger family of TIM molecules with a total of four functional active TIM proteins. TIM-1 has been linked to a number of inflammatory and autoimmune disorders[16]. Our research group had initially identified a protective role for TIM-1, since administration of mice with a TIM-1 inhibiting antibody resulted in exacerbated atherosclerosis[17]. After this publication, others showed that TIM-1 was also an important protein for the induction and maintenance of IL-10^{+} B cells[18–20]. Moreover, it was shown that a different inhibiting TIM-1 antibody resulted in increased regulatory B cells and attenuated atherosclerosis, suggesting a proatherogenic role for TIM-1[21]. These discrepancies were later clarified when it was demonstrated that depending on antibody affinity or ligand density, TIM-1 could act both as an inhibitory and as a stimulatory coreceptor. This led us to investigate atherosclerosis development in a mouse model in which TIM-1 signaling was impaired. We describe in chapter 4 that compared to wild-type mice, these TIM-1$^{\Delta mucin}$ mice show a strong deficit in IL-10 production by B cells, which was associated with a significant increase in viable leukocytes. Furthermore, a specific reduction in Th2 cells and Th2-associated cytokines caused a significant shift towards a more Th1-dependent immune response in TIM-1$^{\Delta mucin}$ mice. Taken together, these immune effects resulted in exacerbated atherosclerosis both in the aortic root and the aortic arch. This work identified that the general contribution of TIM-1 during atherosclerosis development is protective, potentially due to its importance for regulatory B cells.

In chapter 6 we targeted another coreceptor, B- and T-lymphocyte attenuator (BTLA). As the name implies, BTLA is an inhibitory coreceptor which is expressed on both B and T cells. Our work, however, identified that BTLA is predominantly expressed on B cells in atherosclerotic patients and mice. Moreover, we noticed that BTLA was most abundantly expressed on a particular B cell subset, the follicular B2 cells. Given the proatherogenic role of these follicular B2 cells and the inhibitory function of BTLA, we aimed to treat atherosclerosis with an agonistic BTLA antibody. This resulted in a drastic reduction of follicular B2 cells in mice treated with the BTLA agonist. We further investigated the B cell population and found that BTLA stimulation led to a significant increase in the number of regulatory B cell subsets and IL-10^{+} B cells. Similarly, other atheroprotective subsets such as marginal zone B cells and B1 cells were also increased in mice that received the BTLA antibody. Furthermore, the CD4^{+} T cells response was skewed towards a regulatory phenotype. These strong immune effects eventually resulted in a significant decrease in

lesion formation in mice treated with the BTLA antibody. We further investigated whether BTLA agonism also affected established lesions and found that treatment with the BTLA antibody increased lesion stability.

In conclusion, the data described in chapter 4 and 6 underline the central role of co-receptors in the immune system and atherosclerosis. Co-receptors are extensively investigated but often in relation to T cells or dendritic cells. We have shown here that modulation of co-receptors on B cells can also greatly influence atherosclerosis.

Future perspectives

Since the initial proposition of the inflammation theory in atherosclerosis, the research community has made big strides forward. At this point, we are at the onset of clinical use of anti-inflammatory therapy in cardiovascular disease. The recent CANTOS trial showed that antibody-mediated inhibition of interleukin 1β significantly lowered cardiovascular risk independent of lipid-level lowering[22]. This proved the inflammation theory and might pave the way for future clinical trials focusing on anti-inflammatory therapy in cardiovascular disease.

There are many hurdles that have to be overcome before preclinical targets can be validated clinically for their therapeutic effect in atherosclerosis. Hence, it is likely that B cell modulating therapies that are already accepted for the treatment of other disorders will be the first options for clinical use in atherosclerosis. Rituximab, a monoclonal antibody against CD20, depletes B cells and is already registered for clinical use for the treatment of rheumatoid arthritis (RA). Given that CD20-mediated B cell depletion in pre-clinical models of atherosclerosis results in reduced disease[23], rituximab could potentially also be used in atherosclerosis patients. Patients with RA have an increased risk of cardiovascular events, hence, some pilot studies have already investigated the effect of rituximab treatment in RA patients on cardiovascular parameters. In general, short term treatments with rituximab led to decreased carotid intima-media thickness and an overall improvement of cardiovascular parameters, suggesting that this indeed could be a beneficial treatment for atherosclerosis patients[24,25]. Another B cell-depleting therapy, belimumab, blocks B cell activating factor (BAFF) and is now accepted for the use in patients with systemic lupus erythematosus (SLE). Mice deficient for the receptor for BAFF (BAFFR) or treatment of mice with a BAFFR blocking antibody results in attenuated atherosclerosis[26–28], suggesting that blockade of the BAFF/BAFFR pathway is indeed a potential target for the treatment of atherosclerosis. However, a recent study showed that antibody

blockade of BAFF actually exacerbated atherosclerosis[29]. Although the authors also show that this was due to B cell independent protective effects of BAFF, these data do indicate that a more cautious approach is necessary when exploring BAFF blockade in clinical cardiovascular settings. Other B cell modulating therapies that are currently being tested in clinical trials include the use of CD19-mediated B cell depletion. In contrast to CD20, CD19 is also expressed on some antibody-producing plasma cells[30]. Since increased autoantibodies are a detrimental characteristic in many auto-immune disorders, anti-CD19 antibodies represent an interesting option for these disorders. In atherosclerosis, however, the role of IgG antibodies remains controversial and anti-CD19 could also reduce atheroprotective IgM titers. All of these clinically available options are aimed to reduce the total B cell population, which might lead to potential adverse effects, such as increased susceptibility to infections or reduced vaccination efficacy. Additionally, as discussed before, there is a large diversity in the effects of different B cell subsets on atherosclerosis.

Ideally, therapies should focus on specifically reducing atherogenic subsets and/or expansion of atheroprotective subsets. In this thesis we have shown four potential B cell options for the treatment of atherosclerosis which could potentially achieve these effects. For instance, we have shown that an agonistic BTLA antibody specifically reduced B2 cells, while leaving atheroprotective B cell subsets unaffected. We also showed that in cardiovascular patients, the BTLA expression is similar to that of atherosclerotic mice. We have compared the effects of our BTLA antibody with CD20-mediated B cell depletion and found the BTLA antibody superior in terms of influencing the $CD4^+$ T cell response. Hence, the positive results found with rituximab on cardiovascular parameters, could potentially be improved with the use of a humanized agonistic antibody for BTLA. Another target that we discussed in this thesis and that could be targeted with the use of an antibody is TIM-1. We previously showed that a blocking TIM-1 antibody resulted in exacerbated atherosclerosis[17], which is in line with increased lesion development in TIM-$1^{\Delta mucin}$ mice. Therefore, an agonistic TIM-1 antibody should be considered for the treatment of atherosclerosis, which indeed resulted in increased IL-10^+ B cells and ameliorated atherosclerosis in a preclinical mouse model[21].

Most novel biological treatments are based on antibodies, however nowadays, cellular therapy is also an increasingly used option for the treatment of a number of disorders. For instance, there are multiple ongoing clinical trials with regulatory T (Treg) cell therapy in organ transplantation surgeries[31]. They have been able to isolate and generate human Tregs under GMP conditions with similar isolation (microbeads) and culture methods as described in our studies. Our work with IL-10^+ or

PD-L1^{hi} B cells describe preclinical proof-of-concepts but it could be envisioned that experiments focusing on translational aspects, may lead to the actual clinical use of regulatory B cell therapy. For instance, it has recently been shown that patients with atherosclerosis have a lower frequency of circulating IL-10^{+} B cells[32]. Such clinical studies strengthen the rationale for therapies aimed to increase these regulatory B cells and remain of great interest. Furthermore, as we demonstrated in our BTLA study, to increase the clinical relevance of adoptive regulatory B cell transfers it would be interesting to investigate if we could induce plaque regression or induce plaque stabilization with such treatments.

Taken together, in the chapters presented here we have shown that each approach to modulate the B cell response had significant effects on other immune pathways in particular on CD4^{+} T cells, without significant impact on the antibody-producing capacities of B cells. This supports the notion that B cells have many antibody-independent effects and that these processes can strongly influence atherosclerosis. As depicted in figure 1, B cell research in the context of atherosclerosis is still lagging behind research into CD4^{+} T cells, however it seems like the research community is catching up and the therapeutic value of modulation of B cell responses in atherosclerosis receives increasingly more attention nowadays. The work presented in this thesis is proof of that sentiment and provides a foundation for further research into B cells and atherosclerosis.

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Nederlandse samenvatting

Introductie

Cardiovasculaire aandoeningen of hart- en vaatziekten zijn de overkoepelende categorie waaronder welbekende complicaties zoals een hartaanval, beroerte of hartritmestoornissen behoren. De grote maatschappelijke impact die hart- en vaatziekten hebben volgt onder meer uit de schatting van de Wereldgezondheidsorganisatie dat jaarlijks meer dan 15 miljoen mensen overlijden aan het gevolg van dergelijke aandoeningen. Nederlandse cijfers laten zien dat het ook in ons eigen land nog steeds een groot probleem vormt. Volgens de Hartstichting zijn er in Nederland ruim 1.4 miljoen patiënten met enige vorm van hart- en vaatziekten en wat wellicht nog meer tot de verbeelding spreekt, is dat er geschat wordt dat er dagelijks zo'n 100 mensen sterven aan een cardiovasculaire aandoening in Nederland. Niettemin is dit aantal tussen 1980 en 2016 al sterk gedaald doordat we beter zijn geworden in het managen van de onderliggende oorzaak; slagaderverkalking.

Atherosclerose

Slagaderverkalking of *atherosclerose* is een aandoening waarbij er verdikkingen plaatsvinden in de vaatwand, deze worden over het algemeen *laesies* of *plaques* genoemd. In eerste instantie wordt een dergelijke laesie veroorzaakt door schade aan de vaatwand, wat door meerdere risicofactoren verergert of versneld kan worden, bijvoorbeeld alcoholgebruik, roken, overgewicht en hoge bloeddruk. Schade aan de vaatwand in combinatie met een hoog cholesterolgehalte leidt ertoe dat het cholesterol zich gaat ophopen binnen de vaatwand. Vaak spreekt men hier wel over het slechte cholesterol genaamd lage-dichtheids lipoproteïne, oftewel *LDL*. Het LDL wat zich binnen de vaatwand bevindt zorgt ervoor dat de cellen die de vaatwand bekleden, endotheelcellen, geactiveerd raken. Deze cellen brengen vervolgens bepaalde signaaleiwitten tot expressie die immuun cellen vanuit de bloedbaan de vaatwand in kunnen trekken. In eerste instantie zijn dit voornamelijk monocyten, die onder invloed van verschillende factoren binnenin het weefsel verder ontwikkelen tot macrofaag. Ondertussen ondergaat het cholesterol binnen in de vaatwand verschillende veranderingen waardoor het cholesterol verandert in geoxideerd cholesterol of *oxLDL*. Deze verandering van het LDL tot oxLDL leidt ertoe dat het immuunsysteem dit cholesterol gaat beschouwen als lichaamsvreemde stof. Het is geen toeval dat het immuunsysteem als eerste reageert via macrofagen. De letterlijke vertaling vanuit het oude Grieks voor deze macrofagen is namelijk veelvraten. Deze naam gaat gepaard met hun functie, namelijk het opeten of fagocyteren van externe stoffen. Dit is exact wat de macrofagen in de vaatwand doen, zij fagocyteren het

schadelijk oxLDL en ruimen op deze manier het bloedvat op. Macrofagen die grote hoeveelheden vetten zoals cholesterol gefagocyteerd hebben, hebben onder de microscoop een “schuimig” uiterlijk en worden daarom ook wel *schuimcellen* genoemd. In deze fase van atherosclerose is de verdikking van de vaatwand nog minimaal en over het algemeen compleet zonder symptomen voor de patiënt. Dit soort laesies, in deze fase *fatty streaks* genoemd, komen vaak ook al voor in jongvolwassenen en horen in zeker zin tot een normaal ouderdomsverloop.

Echter, zolang de patiënt geen actie onderneemt om het hoge cholesterolgehalte te verminderen, blijft het zich ophopen in de vaatwand, wat ertoe leidt dat de macrofagen niet meer om kunnen gaan met de grote hoeveelheid oxLDL. Vanaf dit punt wordt het initieel beschermende karakter van de immuunreactie meer en meer een schadelijk proces. Het begint bij de macrofagen die uiteindelijk zo vol zitten met oxLDL, dat ze hierdoor afsterven via een proces genaamd *apoptose*. Cellen die sterven geven allerlei stoffen af die zich normaal gesproken exclusief binnen een cel bevinden. Zodra deze stoffen buiten een cel komen, herkennen immuun cellen dit als een schadelijk en lichaamsvreemd proces en sturen ze extra immuun cellen hierop af. De schuimcellen zelf en de endotheelcellen scheiden ondertussen ook signaalstoffen uit om extra hulp in te roepen van het immuunsysteem. Het immuunsysteem is dan al niet meer in staat om de ophoping van oxLDL tegen te gaan en juist door de komst van andere immuun cellen zoals, neutrofielen, dendritische cellen, B cellen en T cellen, neemt de verdikking van de vaatwand alsmaar toe. Door de heftige immuunreactie raken ook de omliggende gladde spiercellen van de vaatwand geactiveerd. Deze cellen migreren richting de laesie en vormen een beschermende laag over de ophoping van vetten en immuun cellen, zodat dit niet blootgesteld kan worden aan het bloed. Ze produceren daarnaast ook collageen strengen die er voor zorgen dat al het geaccumuleerde materiaal op zijn plek blijft. Deze gladde spiercellen zorgen in eerste instantie dus voor stabilisatie van de laesie. Stabiele laesies leiden vaak nog niet tot grote complicaties, maar de vernauwing van het bloedvat (*stenose*) kan op zichzelf al leiden tot problemen. Een dergelijke stenose kan dusdanig ernstig worden dat het weefsel achter de vernauwing onvoldoende doorbloed raakt of *ischemisch* wordt. In minder zware vormen kan dit leiden tot druk op de borst (angina pectoris) of bijvoorbeeld kortademigheid.

Zwaardere complicaties zijn vaak het gevolg van een instabiel geraakte laesie, wat veroorzaakt wordt door meerdere processen. Ten eerste ontstaat er zodra er meer en meer immuun cellen in de vaatwand sterven een zogenaamde *necrotische kern*. De aanwezigheid van deze ophoping van kapotte cellen en afvalstoffen draagt bij aan het instabiel worden van de laesie. Ten tweede wijzen veel wetenschappelijke

studies er op dat de gladde spiercellen hun stabiliserende functie langzamerhand verliezen doordat de beschermende laag en de collageen strengen weer worden afgebroken. Dit alles kan leiden tot het scheuren van de laesie waarbij de inhoud aan het bloed bloot gesteld wordt. Hierbij vormt zich dan een stolsel of *trombus* dat de bloedtoevoer naar het achterliggende weefsel compleet kan afsluiten. Afhankelijk van de locatie kan dit leiden tot bijvoorbeeld een hart- of herseninfarct.

Bij acute complicaties zijn er verschillende opties om de bloedtoevoer te herstellen. Eén daarvan is om te dotteren. Hierbij wordt er gebruik gemaakt van een soort ballon die opgepompt wordt binnen het bloedvat om zodanig weer voldoende bloed naar het achterliggende weefsel toe te laten. In veel gevallen wordt er dan ook gebruik gemaakt van een stent (een soort veertje) dat in het bloedvat wordt geplaatst dat voorkomt dat het vat zich weer sluit na het dotteren. Indien de slagader ernstig vernauwd of compleet afgesloten is, kan er ook gekozen worden voor een zogenaamde bypass operatie. Hierbij wordt een ader uit een ander deel van het lichaam verwijderd en wordt hiermee een omleiding van de bloedtoevoer naar het afgesloten weefsel gemaakt. Een andere operatieve behandeling houdt in dat de complete laesie verwijderd wordt door middel van een endarterectomie. Al deze invasieve methoden hebben als nadeel dat ze vaak door de chirurgische handelingen ook schade aan het bloedvat leveren. Deze schade heeft weer ten gevolg dat de kans op een nieuwe vernauwing, *restenose*, zeer groot is. Hierom en vanwege de niet geringe risico's die gepaard gaan met deze ingrepen, blijft het voorkomen van acute cardiovasculaire complicaties van zeer groot belang.

Hiertoe is een verandering in levensstijl is vaak een eerste optie, gezien veel patiënten in de huidige Westerse maatschappij blootgesteld zijn aan een groot aantal risicofactoren (alcoholgebruik, roken, sedimentaire levensstijl, overgewicht). Ten tweede wordt er vaak gebruikt gemaakt van cholesterol verlagende middelen, meestal in de vorm van statines. Statines remmen de aanmaak van cholesterol in de lever en zijn de reden dat er een sterke afname in cardiovasculair-gerelateerde sterfgevallen zijn sinds de jaren 80. Er is gebleken dat ze het risico op hart- en vaatziekten met circa 25% verminderen. Vrij recent zijn er nieuwe middelen op de markt gekomen die cholesterol niveaus kunnen verminderen via het remmen van PCKS9. Dit eiwit zorgt voor de afbraak van de receptor voor het slechte cholesterol, de LDL receptor. Door dit eiwit te blokkeren, zijn er meer LDL receptoren die LDL wegvangen uit de bloedsomloop en waardoor de cholesterol concentratie wordt verlaagd. Ondanks de grote successen die behaald zijn met cholesterol verlagende middelen, hebben ze als groot nadeel dat ze bestaande laesies niet of nauwelijks beïnvloeden. Over het algemeen krijgen patiënten pas symptomen (en dus behan-

deling) op het moment dat de laesies al vrij groot zijn geworden en er al een heftige immuunrespons plaatsvindt binnen het bloedvat. Naast de cholesterolverlagende middelden is het immuunsysteem daarom een tweede belangrijke pijler waarop men therapeutisch in kan grijpen bij aderverkalking. Deze ontstekingstheorie heeft in de afgelopen decennia veel aandacht gekregen in de onderzoekswereld. Een belangrijke mijlpaal werd vrij onlangs bereikt toen een grote klinische studie aantoonde dat het remmen van de ontstekingsfactor interleukine-1β resulteerde in 15% minder sterfgevallen als gevolg van een cardiovasculaire complicatie. Dit onderzoek bewees dat de ontstekingstheorie van aderverkalking ook daadwerkelijk succesvol vertaald kon worden naar een klinische therapie. Echter, vergeleken met het onderzoek naar cholesterolverlagende middelen, staat het onderzoek naar het immuunsysteem voor de behandeling van hart- en vaatziekten nog in de kinderschoenen en valt er nog veel winst te boeken. Dit onderstreept duidelijk het belang van verder onderzoek naar de invloed van specifieke immuun cellen en ontstekingsfactoren op de ontwikkeling van atherosclerose.

Het immuunsysteem

Het immuunsysteem is de belangrijkste verdediging van het lichaam tegen externe en interne ziekteverwekkers of schadelijke processen. Een van de belangrijkste taken hierbij is om onderscheid te kunnen maken tussen lichaamseigen en niet-lichaamseigen cellen en moleculen. De wijze waarop immuun cellen dit onderscheid kunnen maken verdeelt het immuunsysteem grofweg in twee takken: aangeboren of aspecifieke immuniteit en verworven of specifieke immuniteit.

Vrijwel alle organismen bezitten een vorm van het aangeboren immuunsysteem. Deze aspecifieke immuniteit herkent met behulp van speciale receptoren patronen en structuren die vaak voorkomen op bacteriën en/of virussen, maar kan hierbij geen onderscheid maken tussen specifieke pathogenen. Het grote voordeel van een dergelijk afweersysteem is dat het snel kan reageren op potentieel gevaar. De monocyten en macrofagen die al eerder benoemd waren zijn onderdeel van het aangeboren immuunsysteem, evenals de dendritische cel. Zowel macrofagen als dendritische cellen controleren de extracellulaire ruimtes en kunnen moleculen die niet in het lichaam thuis horen fagocyteren en zijn op die manier onderdeel van de eerstelijnsafweer. Daarnaast hebben zij nog een tweede zeer belangrijke functie, namelijk verbinding maken met cellen van de verworven immuniteit. Nadat een macrofaag of dendritische cel een bepaald niet-lichaamseigen molecuul heeft gefagocyteerd, zorgt deze cel ervoor dat (gedeeltes van) dit molecuul aan de buitenkant

van zijn celmembraan op een herkenbare manier gepresenteerd worden aan cellen van de specifieke afweer. Daarom worden macrofagen en dendritische cellen ook wel antigeen-presenterende cellen genoemd.

Immuun cellen van de specifieke afweerreactie hebben over het algemeen hulp nodig van antigeen-presenterende cellen en deze reactie komt daarom ook langzamer op gang dan de aangeboren afweer. De belangrijkste cellen van deze tak van het immuunsysteem zijn B- en T-lymfocyten. Deze B en T cellen zijn zeer gespecialiseerde ontstekingscellen zodat elk van deze cellen maar één specifiek molecuul kan herkennen. In de ontwikkeling van deze cellen wordt elke B of T cel die een lichaamseigen molecuul herkent verwijderd, waardoor er alleen B en T cellen overblijven die lichaamsvreemde antigenen herkennen. Zodra een antigeen-presenterende cel moleculen presenteert op de buitenkant van zijn celmembraan en een B of T cel dat specifieke antigeen herkent, raakt de cel geactiveerd. Deze activatie leidt er voornamelijk toe dat de cel zich veelvuldig gaat delen, zodat er een grote populatie aan immuun cellen ontstaat dat dit antigeen herkent. Geactiveerde T cellen zorgen voornamelijk voor de afweer tegen pathogenen die zich binnenin cellen bevindt door ontstekingsfactoren uit te scheiden of door geïnfecteerde cellen direct kapot te maken.

B cellen daarentegen scheiden antistoffen uit die in het bloed circuleren en het specifieke antigeen kunnen neutraliseren. Nadat alle gevaarlijke antigen opgeruimd zijn door het immuunsysteem differentieert een kleine groep van specifieke B en T cellen zich tot geheugencellen. Deze populatie van geheugencellen kan zeer lang overleven in het lichaam en zorgt ervoor dat bij een volgende infectie met dit antigeen de specifieke afweerreactie veel sneller en sterker zal zijn. Dit geheugen van het specifieke afweersysteem vormt de basis van vaccinaties, waarbij een klein, niet gevaarlijk, gedeelte van een pathogeen wordt geïnjecteerd. De patiënt raakt hier niet ziek van, maar creëert wel een populatie geheugencellen zodat bij een daadwerkelijke infectie, de patiënt beschermd is door een effectieve immuunreactie.

Dit proefschrift

Centraal in deze thesis staat de ontstekingstheorie van atherosclerose, waarbij ervan uit gegaan wordt dat het remmen van de immuunreactie een gunstig effect zal hebben op het ziekte verloop. Het belangrijkste celtype dat dit proefschrift daarbij behandeld is de B cel. Het klassieke kenmerk van B cellen is zogezegd de productie van antistoffen of antilichamen. In de laatste decennia is de niet-klassieke rol van B

cellen desalniettemin steeds duidelijker geworden. Zo zijn B cellen bijvoorbeeld ook zeer goed antigeen-presenterende cellen en kunnen ze een grote verscheidenheid aan ontstekingsmediatoren uitscheiden. Daarnaast zijn er ook nieuwe type B cellen gevonden, waarvan het onbekend was welke rol deze speelden in de pathofysiologie van aderverkalking. In dit proefschrift is geprobeerd op verschillende manieren B cellen te beïnvloeden waarbij we ons geconcentreerd hebben op de nieuwe type B cellen en niet-klassikale functies om te onderzoeken wat hiervan het effect zou zijn op aderverkalking,

In **hoofdstuk 2** hebben we het laatste onderzoek naar de rol van B cellen bij aderverkalking samengevat en zijn we specifiek ingegaan op potentiele functies van nieuwe type B cellen. B cellen kunnen over het algemeen in twee verschillende types onderscheiden worden: B1 en B2 cellen. B2 cellen komen veruit het meeste voor en ontwikkelen zich in het beenmerg en circuleren vervolgens in het bloed en lymfoïde organen, zoals de milt. Hier ontwikkelen de meeste B2 cellen zich verder tot folliculaire B cellen, terwijl een kleinere populatie differentieert in marginale zone B cellen. Deze verschillende type B2 cellen reageren op andere stimuli en hebben ook een verschillend effect op aderverkalking. Alle B cellen bezitten een specifieke receptor die grotendeels bestaat uit een antistof dat zich in het celmembraan bevindt. Wanneer deze receptor zijn specifieke antigeen herkent en bindt, is dat het eerste activatiesignaal voor een B cel. De B cellen fagocyteren vervolgens het gebonden antigeen en presenteren gedeeltes ervan aan de buitenkant van het celmembraan. Voor de activatie van folliculaire B cellen is het noodzakelijk dat een naburige T cel ook het gepresenteerde antigeen herkent. Deze T cel zal vervolgens ook geactiveerd raken en een verscheidenheid aan mediatoren en stoffen uitscheiden die het tweede activatiesignaal vormen voor de B cel. De B cellen zullen vervolgens gaan delen en via complexe selectieprocedure, waarbij T cellen wederom een belangrijke rol spelen, ontstaat er een populatie van B cellen met een receptor die zeer specifiek en met een hoge affiniteit aan het antigeen kan binden. Deze populatie ondergaat vervolgens een verandering waarbij het grootste gedeelte differentieer in plasma cellen, wat feitelijk cellen zijn die zeer grote hoeveelheden antistoffen uitscheiden om het antigeen te neutraliseren. De overige B cellen zullen differentiëren in geheugen B cellen. In aderverkalking nog niet volledig bekend wel antigeen verantwoordelijk is voor de immuunreactie. Er wordt vaak aangenomen dat het belangrijkste eiwit in LDL, het ApoB100 eiwit, het meest bijdraagt aan de immuunreactie. Onderzoek heeft uitgewezen dat er inderdaad antistoffen tegen ApoB100 gemaakt kunnen worden, maar er is nog geen duidelijke consensus of dat deze antistoffen bijdragen aan verslechtering van de ziekte. Dit komt mede doordat er een grote verscheidenheid aan verschillende type antilichamen zijn. Folliculaire B

cellen scheiden met name antilichamen uit van het IgG type. Alhoewel hier ook nog geen sluitend bewijs voor is, wijst de meeste data er op dat deze type antistoffen atherosclerose verergeren. In ieder geval is het vanuit onderzoek wel duidelijk dat folliculaire B cellen bijdragen aan de ontwikkeling van aderverkalking, aangezien verwijdering van deze B cellen leidt minder aderverkalking optreedt. Niettemin is het exacte mechanisme hierachter nog niet geheel duidelijk.

Terwijl folliculaire B cellen afhankelijk zijn van de hulp van T cellen, kunnen marginale zone B cellen ook reageren op antigeen zonder T cellen. Onlangs is gebleken dat deze marginale zone B cellen juist beschermend werken bij aderverkalking. Ditzelfde geldt voor de B1 cellen. Deze B1 cellen ontwikkelen zich niet vanuit het beenmerg zoals B2 cellen, maar vanuit de lever van de foetus. Een belangrijke eigenschap van B1 cellen is dat ze ook zonder antigeen grote hoeveelheden antistoffen uitscheiden van het type IgM. Deze antilichamen kunnen een groot scala aan antigenen binden, waaronder oxLDL. Onderzoek wijst uit dat neutralisatie van oxLDL door deze natuurlijke antilichamen een groot onderdeel is van de beschermende functie achter B1 cellen.

Naast deze indeling in B1 en B2 cellen zijn we in hoofdstuk 2 ingegaan op nieuwe subsets van B cellen, die vaak niet geheel binnen deze B1/B2 classificatie vallen. Het belangrijkste subtype wat wij hier behandeld hebben is de zogenaamde regulatoire B cel. Het immuunsysteem heeft bepaalde mechanismen ingebouwd waarbij het zichzelf kan reguleren in het geval het gevaar geweken is of indien er geen actieve immuunrespons gewenst is. Het meest bekend en veruit het meest intensief onderzochte type cel in dit aspect is de regulatoire T cel. Dit type T cel kan door bijvoorbeeld de secretie van de remmende mediatoren zoals interleukine-10 de activatie van overige immuun cellen remmen. In de afgelopen jaren heeft onderzoek aangetoond dat er binnen de B cellen ook een dergelijk type B cel bestaat dat het immuunsysteem kan remmen. Er zijn verscheidene manieren waarop deze B cellen functioneren, maar de bijdrage van deze verschillende functies en type B cellen aan de ontwikkeling van aderverkalking was nog geheel onbekend.

In **hoofdstuk 3** zijn wij daarom gaan onderzoeken wat het effect is van B cellen die interleukine-10 kunnen uitscheiden op de ontwikkeling van atherosclerose. Gezien het overtuigende bewijs dat regulatoire T cellen die interleukine-10 aanmaken beschermend zijn, was onze hypothese dat deze regulatoire B cellen dat ook zouden zijn. Ons eerste experiment bestond uit het observeren van het aantal interleukine-10 producerende B cellen in gezonde en atherosclerotische muizen. We vonden dat de zieke muizen significant minder van deze B cellen hadden dan

gezonde muizen. Omdat dit uitwees dat bij het ontwikkelen van aderverkalking de populatie regulatoire B cellen afnam, besloten wij om te proberen om deze populatie in stand te houden tijdens een tweede experiment. Hiertoe ontwikkelden we een kweekprotocol waarmee we *in vitro* aan grote hoeveelheden van interleukine-10 producerende B cellen konden komen. Deze injecteerden we tijdens de loop van het experiment een aantal keer in één groep muizen. Aan het einde van het experiment vergeleken we het immuunsysteem van deze muizen, met het immuunsysteem van muizen die ofwel geen B cellen hadden gekregen of B cellen die geen interleukine-10 kunnen produceren. De eerste resultaten waren veelbelovend; het immuunsysteem in de muizen die interleukine-10 producerende B cellen kregen was duidelijk geremd. We zagen een afname in het aantal ontstekingscellen, een afname in de T cel activatie en juist een toename in het aantal regulatoire T cellen. Toen we echter de mate van aderverkalking bepaalden zagen we dat dit uiteindelijk niet het volledig gewenste effect had bewerkstelligd. De muizen die waren geïnjecteerd met B cellen die geen interleukine-10 kunnen uitscheiden, hadden een sterke verergering van aderverkalking. Deze observatie was nog volgens de hypothese, aangezien deze niet-interleukine 10 producerende B cellen feitelijk folliculaire B cellen zijn. Niettemin zagen we dat de muizen die de interleukine-10 producerende B cellen hadden ontvangen geen extra bescherming hadden ondervonden; zij toonden dezelfde mate van aderverkalking als muizen die geen B cellen hadden gekregen. Verdere analyse vertoonde tot onze initiële verbazing dat zowel de muizen die de niet-interleukine 10 producerende B cellen als wel de muizen die de interleukine 10 producerende B cellen hadden gekregen, een significante verhoging van cholesterol hadden. Zoals besproken is cholesterol een zeer belangrijke determinant voor de mate van aderverkalking, dus deze bevinding zou kunnen verklaren waarom we ondanks de remming van het afweersysteem geen effect op aderverkalking zagen met de interleukine-10 producerende B cellen. Verdere experimenten suggereerden dat dit effect op cholesterol mogelijk door ons kweekprotocol zou kunnen komen. Uit het gehele hoofdstuk kunnen we concluderen dat interleukine-10 producerende B cellen minder aanwezig zijn in atherosclerotische muizen en dat injectie van deze cellen leidt tot remming van het afweersysteem. Niettemin had dit geen effect op aderverkalking, wellicht doordat deze effecten gemaskeerd werden door een verhoging in cholesterol.

In **hoofdstuk 4** hebben we op een andere manier geprobeerd om interleukine-10 producerende B cellen te beïnvloeden. Eerder onderzoek had aangetoond dat een specifiek eiwit genaamd TIM-1 erg belangrijk was voor de ontwikkeling van B cellen tot regulatoire B cellen. Muizen die dit eiwit missen hebben vrijwel geen interleukine-10 producerende B cellen. Via een speciale virusinjectie hebben wij de

ontwikkeling van aderverkalking in deze muizen die TIM-1 missen (TIM1$^-$) kunnen vergelijken met normale wild-type (WT) muizen. We observeerden inderdaad dat deze TIM1$^-$ muizen een verminderd aantal regulatoire B cellen hadden ten opzichte van WT muizen. Dit ging ook gepaard met een toename van B en T cellen in de milt. Ook de immuunreactie van de T cellen was opgeschoven naar een meer schadelijke response. Gezamenlijk leidde dit uiteindelijk tot een sterke toename van aderverkalking in TIM1$^-$ muizen ten opzichte van WT muizen. Het TIM-1 eiwit bevindt zich niet alleen op B cellen en de verwijdering van TIM-1 in deze muizen kan ook effecten hebben gehad op andere cel populaties. Evengoed suggereert deze data dat TIM-1 een belangrijk eiwit is bij de ontwikkeling van aderverkalking en dat dit effect gemedieerd wordt via regulatoire B cellen.

Een andere regulatoire B cel populatie hebben we in **hoofdstuk 5** onderzocht. Hier hebben we ons geconcentreerd op B cellen die het eiwit PD-L1 hoog tot expressie brengen. Dit eiwit kan binden aan PD-1 op T cellen, wat er vervolgens voort zorgt dat deze T cel geremd wordt in zijn functie. De meeste T cellen die PD-1 tot expressie brengen zijn zogenaamde folliculaire T helper cellen, die hulp bieden aan folliculaire B cellen bij het ontwikkelen tot plasma cellen. Aangezien het bekend is dat deze folliculaire B cellen aderverkalking verergeren, stelden wij dat B cellen met hoge PD-L1 expressie wellicht zouden kunnen leiden tot een vermindering van aderverkalking. Deze PD-L1^{hi} B cellen komen vrij weinig voor daarom zijn wij op zoek gegaan naar een methode om PD-L1 op meer B cellen tot expressie te brengen. Wij vonden dat wanneer B cellen vierentwintig uur werden gestimuleerd met de stof interferon-gamma, dat vrijwel alle B cellen PD-L1 gingen aanmaken en dat het overgrote gedeelte zeer veel PD-L1 tot expressie bracht. Deze cellen hebben we vervolgens zowel *in vitro* als *in vivo* getest om te onderzoeken of ze inderdaad folliculaire helper T cellen konden remmen en in beide situaties ondervonden we dat deze cellen zeer effectief waren hierin. Vervolgens hebben deze cellen geïnjecteerd in muizen om het effect hiervan op aderverkalking te evalueren. Wij zagen toen dat de totale verdikking in de aderen van muizen die de interferon-gamma gestimuleerde B cellen hadden gekregen significant minder was dan muizen die deze B cellen niet hadden gekregen. Vergeleken met muizen die niet-gestimuleerde B cellen hadden gekregen was het verschil net niet statistisch significant, maar ook duidelijk aanwezig. Kortom, stimulatie van B cellen met de stof interferon-gamma resulteerde in hoge expressie van PD-L1 waarmee folliculaire helper T cellen konden worden geremd en aderverkalking werd verminderd.

In **hoofdstuk 6** hebben we een andere manier gebruikt om de B cel reactie te moduleren. Activatie van B en T cellen vereist naast het antigeen vaak nog een twee

signaal via co-receptoren. Dit signaal komt vaak van antigeen-presenterende cellen en kan zowel stimulerend als remmend werken. Een redelijk recent beschreven co-receptor is BTLA (B- and T-lymphocyte attenuator) dat een remmende functie heeft. Deze remmende co-receptoren zijn interessant voor de mogelijke behandeling van aderverkalking, omdat wanneer het lukt deze remmende functie te versterken, het immuunsysteem wordt gedempt en aderverkalking wellicht afneemt. In de eerste experimenten gingen wij onderzoeken op welk type cellen BTLA tot expressie kwam. We zagen zowel in de mens als de muis dat BTLA voornamelijk op B cellen voorkwam en specifiek op de folliculaire B cellen. Daarom kozen wij ervoor om muizen te behandelen met een antilichaam dat bindt aan BTLA en de remmende werking van BTLA in gang zet. Behandeling van muizen met dit specifieke antilichaam zorgde ervoor dat de folliculaire B cellen zeer sterk afnamen, terwijl beschermende B cel populaties zoals de B1 cellen en interleukine-10 producerende B cellen in aantallen niet afnamen. Dit resulteerde feitelijk in dat de B cel populatie overwegend uit beschermende B cellen bestond in de behandelde muizen. Verdere analyse toonde aan dat de gehele immuunrespons en met name de T cel reactie verschoof richting een meer beschermend karakter. Evaluatie van aderverkalking wees uit dat het gebruikte BTLA antilichaam aderverkalking had geremd ten opzichte van geen behandeling of behandeling met een controle antilichaam. In een andere proef bekeken we ook het effect van dit BTLA antilichaam op al bestaande laesies. We ondervonden dat de laesies niet kleiner werden, maar wel stabieler wat evengoed een zeer belangrijke parameter is. In dit hoofdstuk konden we concluderen dat stimulatie van BTLA met een antilichaam er voor zorgt dat folliculaire B cellen worden geremd en dat dit een gunstig effect heeft op de ontwikkeling van aderverkalking.

In **hoofdstuk 7** hebben wij een overkoepelende discussie geschreven over het totale werk wat in dit proefschrift aan bod komt. In het kort, concluderen wij hierin dat het onderzoek naar B cellen en aderverkalking enigszins is achtergebleven in de afgelopen jaren, terwijl er alle redenen toe zijn om dit onderzoek juist wel uit te voeren. De beschreven experimenten in dit proefschrift tonen namelijk aan dat B cellen een belangrijke rol spelen in de immuunreactie die optreedt tijdens aderverkalking en dat wanneer deze rol wordt gemoduleerd er duidelijke effecten optreden. Het bewijs dat hiervoor is aangevoerd in dit proefschrift is overwegend uitgevoerd in muizen en van een observerend karakter. Om deze resultaten te kunnen vertalen naar daadwerkelijke klinische toepassingen zal er in eerste instantie verder onderzoek gedaan moeten worden in kleine patiënt groepen of op humaan weefsel. Dat type onderzoek vergt vaak ook dat er nog meer mechanistische studies in muizen worden gedaan die dieper ingaan op de achterliggende werking van eventuele therapieën. Op dit moment zijn er al middelen voorhanden die B cellen kunnen depleteren of

verwijderen in mensen. Deze worden al toegepast voor de behandeling van met name reuma, maar nog niet voor cardiovasculaire ziekten. Wel zijn er al preliminaire aanwijzingen gevonden dat reuma patiënten die behandeld zijn met een dergelijk middel, minder risico lopen op hart- en vaatziekten. Het is aannemelijk dat deze middelen die alle B cellen remmen of verwijderen als eerste toegepast zullen worden in de klinische setting voor hart- en vaatziekten. Echter laat het bewijs uit de literatuur en in dit proefschrift zien dat het van essentieel belang is om specifieke subsets te remmen dan wel te stimuleren. Daarnaast hebben alle experimenten in deze thesis de B cel reactie beïnvloedt, maar niet of nauwelijks de antilichaam productie van B cellen. Dit benadrukt de grote invloed van niet-klassikale functies van B cellen in het algemeen en in de context van atherosclerose.

Het doet me deugd om te zien dat er tijdens het werken aan deze thesis langzamerhand weer meer en meer publicaties verschijnen die eenzelfde invalshoek hebben als het werk in dit proefschrift. Dit geeft aan dat de rol van B cellen in aderverkalking in de onderzoek community meer aandacht krijgt en de experimenten in dit proefschrift zijn een goede basis om hier in de toekomst meer onderzoek naar te doen.

Scientific publications

PhD Portfolio

Curriculum vitae

Scientific publications

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PhD Portfolio

Courses

2015 LACDR Data management course
2015 On being a scientist (ethics in science)
2014 Introduction to teaching and supervision
2014 Communication in Science
2014 PhD Introductory course on drug research

Presentations

2018 International Symposium on Atherosclerosis, Toronto, Canada
2018 Scandinavian Society for Atherosclerosis Research, Humblebæk, Denemarken
2018 LACDR spring symposium, Leiden, Nederland
2017 Scandinavian Society for Atherosclerosis Research, Humblebæk, Denemarken
2017 LACDR spring symposium, Leiden, Nederland
2017 Rembrandt symposium, Noordwijkerhout, Nederland
2016 LACDR spring symposium, Leiden, Nederland
2016 Scandinavian Society for Atherosclerosis Research, Humblebæk, Denemarken
2015 Rembrandt symposium, Noordwijkerhout, Nederland
2015 LACDR spring symposium, Leiden, Nederland
2014 Rembrandt symposium, Noordwijkerhout, Nederland
2014 LACDR spring symposium, Leiden, Nederland

Teaching

2018 Supervision of MSc internship – Sanne kroon
2017 BSc course Therapeutic modulation of atherosclerosis
2017 Supervision of BSc internships – Emma Pool and Tamar Woudenberg
2017 Supervision of MSc internship – Lars Koeken
2016 BSc course Therapeutic modulation of atherosclerosis
2016 BSc course Geneesmiddel Toediening en afgifte
2016 Supervision of BSc internships – Louise Tambyrajah and Mallory van den Heuvel
2016 Supervision of MSc internship – Bianca Suur
2015 Supervision of BSc internships – Bianca Suur and Arlette Quaedvlieg
2015 BSc course Therapeutic modulation of atherosclerosis
2015 BSc course Geneesmiddel Toediening en afgifte
2014 Supervision of BSc internship – Rianne de Zwart
2014 BSc course Geneesmiddel Toediening en afgifte
2014 BSc course Therapeutic modulation of atherosclerosis

Curriculum Vitae

Hidde Douna werd geboren op 16 december 1988 in Hoorn. In juni 2007 behaalde hij zijn gymnasium diploma *cum laude* aan het Stedelijk Gymnasium te Leiden. In datzelfde jaar begon hij aan de studie Natuurwetenschappen & Innovatiemanagement aan de Universiteit Utrecht. Hij behaalde zijn propedeuse in 2008 en besloot toen verder te gaan met de bachelor Farmacie, eveneens aan de Universiteit Utrecht. Hij behaalde de Bachelor of Science graad *cum laude* in juli 2011 en vervolgde zijn opleiding met de master Drug Innovation. Van januari tot september 2012 deed hij zijn eerste masterstage bij het Utrecht Institute for Pharmaceutical Sciences onder begeleiding van dr. C. Rietveld en prof. dr. A. Kraneveld. Deze stage werd afgesloten met een verslag getiteld: "Emerging concepts of the enteric nervous system: Toll-like receptors and Parkinson's disease". Van januari 2013 tot juli 2013 voerde hij een tweede wetenschappelijke stage uit waarvan drie maanden bij het Rush University Medical Center in Chicago. De titel van het onderzoeksproject was: "Rotenone in Parkinson's Disease: from gut to brain and back again" en heeft geleid tot een publicatie in het blad *Nutritional Neuroscience.* Zijn thesis heeft hij onder begeleiding van prof. dr. J. Kuiper uitgevoerd aan de Universiteit Leiden getiteld: "Current state and optimizations for peptide-based vaccines against atherosclerosis". De wetenschappelijke stages en thesis hebben geleid tot het behalen van de Master of Science graad *cum laude* in maart 2014.

Van 2008 tot 2014 heeft hij eveneens deelgenomen aan het *honours programme* vanuit de studie Farmacie. In het kader hiervan heeft hij onder andere in samenwerking met de Parkinson Vereniging een wetenschappelijke studie uitgevoerd dat geleid heeft tot een publicatie in het blad *The Open Pharmacology Journal.*

Van februari 2014 tot oktober 2018 was hij als promovendus werkzaam bij de afdeling BioTherapeutics van het Leiden Academic Centre for Drug Research onder begeleiding van dr. A.C. Foks, dr. G.H.M. van Puijvelde en prof. dr. J. Kuiper. Voor het presenteren van zijn onderzoek ontving Hidde in 2017 zowel de prijs voor beste orale presentatie op het Rembrandt Symposium als de prijs voor beste wetenschappelijke poster op de Annual Meeting van de Scandinavian Society for Atherosclerosis Research.

Sinds 15 maart 2019 is hij in dienst van Novartis Pharma B.V. en werkzaam in het cardio-metabolic team als Medical Scientific Liaison.