B cell modulation in atherosclerosis

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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).

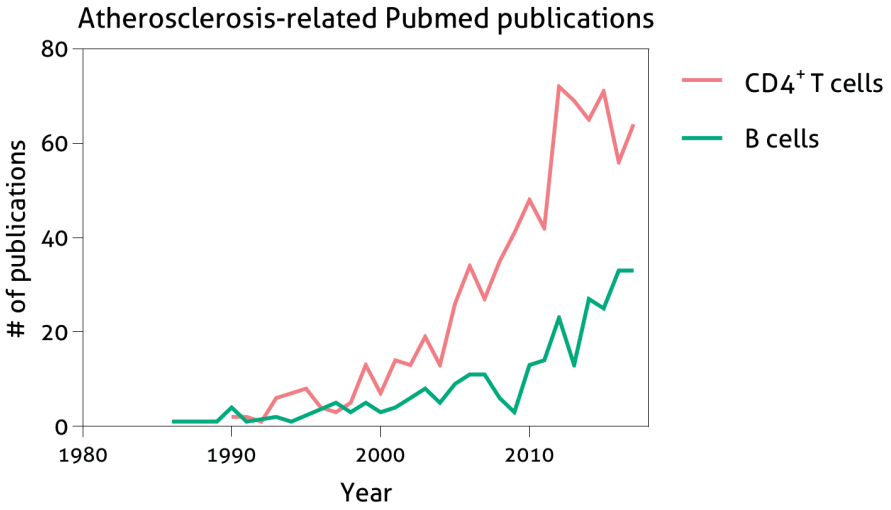

Figure 1. Number of indexed articles in Pubmed. A basic Pubmed search for "CD4" or "B cells" together with "atherosclerosis" was performed (24/10/18). The number of resulting publications are plotted (y-axis) versus the year of publication (x-axis).

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.

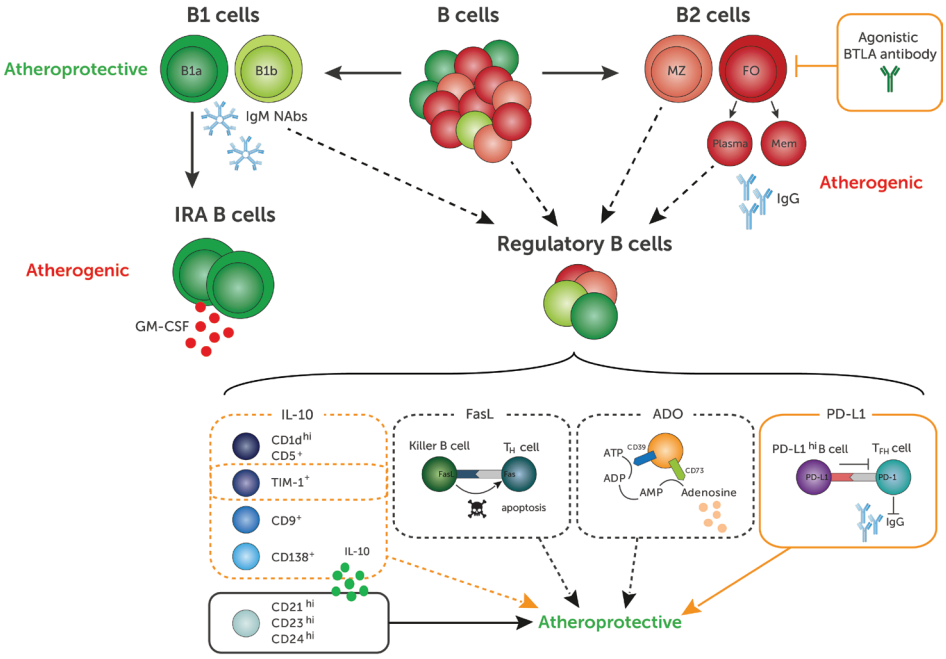

Figure 2. Overview of the main B cell subsets and their known and putative effects on atherosclerosis. Orange highlighted sections indicate the investigated B cell subsets in this thesis.

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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