CD8+ T-cells in Atherosclerosis: mechanistic studies revealing a protective role in the plaque microenvironment

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General Discussion and Perspectives

1. Immunomodulatory treatments for atherosclerosis

Cardiovascular diseases (CVD) remain a major concern for global health, as they contribute to 31% of all deaths worldwide [1]. The main underlying pathogenic process that drives CVD is atherosclerosis, a chronic inflammatory process that results in the buildup of cholesterol-rich plaques within the arteries [2]. As lipids are such an important driving force behind the disease process, current therapeutic interventions focus on the lowering of the plasma cholesterol levels via the use of statins [3] or by inhibiting protein convertase subtilisin/kexin type 9 (PCSK9) [4]. However, treatment with these lipid-lowering drugs is not effective in all patients [5], and even in patients that achieve low cholesterol levels, cardiovascular events still occur [6]. This illustrates that there is a need for innovative treatments that improve cardiovascular outcomes. Recently, there has been increased interest in targeting the immune component of atherosclerosis. The REMOVAL (REducing with MetfOrmin Vascular Adverse Lesions) trial assessed the potential beneficial effect of treatment with metformin, an anti-diabetic drug, on cardiovascular outcomes in patients with type 1 diabetes [7]. Metformin not only can help restore blood glucose levels via activation of AMP-activated protein kinase, but also inhibits nuclear factor κB-mediated inflammatory signaling [8]. Treatment with metformin reduced the maximal common carotid artery intima-media thickness [7], a measure for atherosclerotic plaque size, suggesting that targeting the immune system may be of use in cardiovascular risk management. Indeed, observational studies have shown that in psoriatic arthritis patients, treatment with monoclonal antibodies that block the function of the inflammatory cytokine TNF-α reduces the development of carotid atherosclerotic plaques [9]. There is also a reduced incidence of cardiovascular events in rheumatoid arthritis patients treated with TNF-α-blocking medication [10]. However, blocking the function of TNF-α in cardiovascular patients using either etanercept in the RENEWAL (Randomized Etanercept Worldwide Evaluation) trial [11], or infliximab in the ATTACH (anti-TNF Therapy Against Congestive Heart Failure) trial [12], did not improve and even adversely affected heart failure. This could indicate that specifically targeted treatments as compared with generalized immune suppression may be needed to increase the effectiveness of anti-inflammatory therapies for the treatment of CVD. Alternatively, this could suggest that anti-inflammatory treatments are only beneficial in a certain subset of patients. In agreement with this, the outcome of the CIRT trial (Cardiovascular Inflammation Reduction Trial) suggests that the effectiveness of anti-inflammatory treatment differs based on the inflammation levels of each patient [13], which therefore may be an important inclusion criterion to consider in similar studies. Besides the blocking of TNF-α, there is the currently ongoing ASSAIL-MI trial (ASSessing the Effect of Anti-IL-6 Treatment in Myocardial Infarction), which investigates the effect of blocking the inflammatory cytokine IL-6 as a potential treatment strategy for atherosclerosis. In addition, the CANTOS (Canakinumab Anti-inflammatory Thrombosis Outcome Study) trial studied the effect of treating patients who previously experienced a myocardial infarction with a monoclonal antibody that blocks the function of the inflammatory cytokine IL-1β, on top of state-of-the art lipid treatment. This resulted in a significantly lower rate of recurrent cardiovascular events compared to placebo treatment, although treatment was also associated with a higher incidence of fatal infection, due to generalized suppression of the immune system [14].

This study shows that there is potential in inhibiting the immune system in atherosclerosis, but emphasizes the need for specifically targeted treatments in order to prevent adverse effects related to immune suppression.

$CD8^+$ T-cells can exert their effector functions in an antigen-specific manner, as they release their cytotoxic molecules upon stimulation of their T-cell receptor (TCR) by binding to an MHC-I molecule that presents their cognate antigen. Because of the highly specific nature of this interaction, targeting these cells is of particular interest for modulating the immune system in atherosclerosis, while preventing non-specific immune inhibition. As $CD4^+$ T-cells have already been studied extensively in the context of atherosclerosis [15–17], some progress towards utilizing or targeting $CD4^+$ T-cells for the treatment of atherosclerosis has been made [18–20], demonstrating proof-of-concept for the use of T-cell therapy in this disease. However, research into the role of $CD8^+$ T-cells in atherosclerosis has lagged behind, and much less is known about their contribution to the pathogenesis of atherosclerosis and particularly how these cells function within the lesion microenvironment. Therefore, it is unclear if they can be targeted for the treatment of atherosclerosis.

In this thesis, we aimed to (1) determine the phenotype and function of $CD8^+$ T-cells in atherosclerotic lesions and (2) assess whether therapeutic targeting of $CD8^+$ T-cells can provide a means of modulating the inflammatory responses in atherosclerosis.

2. $CD8^+$ T-cells exert a protective function in advanced atherosclerotic lesions

Experimental studies have provided conflicting evidence regarding the role of $CD8^+$ T-cells in atherosclerosis development. As reviewed in **Chapter 2**, the presence of $CD8^+$ T-cells in atherosclerotic lesions has been established in both beginning and advanced human atherosclerotic plaques [21, 22]. Early studies using genetic knockout mouse models provide contrasting data regarding the role of $CD8^+$ T-cells in the pathogenesis of this disease, suggesting either a limited function [23, 24] or an atheroprotective one [25]. More recent studies have suggested both pro- and anti-atherogenic functions for $CD8^+$ T-cells, depending on what subset is studied and what stage of the lesions is evaluated. For instance, pro-inflammatory cytokine-producing $CD8^+$ T-cells aggravate lesion development in the initial stages of atherosclerosis development due to increased monopoiesis [26]. This effect is most likely mediated via the inflammatory functions of TNF-α and granzyme B, as the adoptive transfer of IFN-γ-deficient $CD8^+$ T-cells does not affect atherosclerosis development compared to transfer of wild-type $CD8^+$ T-cells, suggesting that the role of $CD8^+$ T-cell derived IFN-γ in the pathogenesis of atherosclerosis is limited. Regarding atheroprotective roles for $CD8^+$ T-cell subsets, immunization of $apoE^{-/-}$ mice with an LDL-derived peptide was shown to induce protective $CD8^+$ T-cell responses, by inducing cytolytic activity towards antigen-presenting cells (APCs) [27, 28]. Adoptive transfer of $CD8^+CD25^+$ regulatory T-cells into $apoE^{-/-}$ mice resulted in reduced lesion development, via inhibition of pro-inflammatory $CD4^+$ and $CD8^+$ T-cell responses [29]. Moreover, a subset of Qa1-restricted regulatory $CD8^+$

T-cells has been described to reduce atherosclerotic lesion development in apoE$^{-/-}$ mice via inhibition of T follicular helper cell function, reducing the formation of germinal centers and immunoglobulin production [30].

We also discussed the published work regarding CD8$^+$ T-cell function in human atherosclerosis in this chapter. Increased CD8$^+$ T-cell numbers in the blood are associated with the incidence of coronary events [31]. Later work has shown that this appears to hold true for the number of activated CD8$^+$ T-cells in particular [32, 33]. In contrast, regulatory CD8$^+$ T-cells were shown to exert anti-atherogenic functions in human patients [34], again stressing the need to differentiate between distinct CD8$^+$ T-cell subsets in order to understand their role in atherogenesis.

Finally, we suggest in this review chapter that targeting the right subset of CD8$^+$ T-cells, for instance via vaccination, might be of therapeutic value for the treatment of atherosclerosis. Indeed, boosting CD8$^+$ T-cell responses can induce increased lysis of APCs and reduce atherosclerotic plaque formation [24, 26]. Moreover, boosting the function of the regulatory CD8$^+$ T-cell responses may prove to be an effective strategy, as these cells have been shown to limit atherosclerosis development [26]. However, several challenges remain to be met before CD8$^+$ T-cell vaccination can be successfully employed in atherosclerosis. First of all, antigens have to be identified against which the CD8$^+$ T-cell response can be directed. LDL-derived proteins are potential candidate antigens, as previous studies have shown the induction of T-cell responses towards ApoB100 epitopes [27, 28, 35]. Secondly, it is important to identify the optimal adjuvant, as these immune-boosting compounds can affect atherosclerosis development [36]. Finally, it is of the utmost importance to avoid inducing the pro-atherogenic CD8$^+$ T-cell subsets discussed above.

Of the papers reviewed in **Chapter 2**, most of the experimental studies have focused mainly on initial lesion development, whereas from a clinical point of view, it is more relevant to study advanced and/or unstable lesions. Patients usually experience symptoms related to severe stenosis (such as chest pain and shortness of breath) when lesions are advanced, and seek medical advice at this stage of the disease. Thus, from a drug development perspective, it is most valuable to understand the function of CD8$^+$ T-cells in the progressed stages of atherosclerosis, as this is when pharmacological intervention is possible.

In **Chapter 3** we set out to investigate how CD8$^+$ T-cells affect the stability and composition of advanced atherosclerotic lesions. First, we investigated whether there was any significant correlation between the percentage of CD8$^+$ T-cells present in human atherosclerotic lesions and other immune cells that are known to be involved in atherogenesis. Interestingly, we observed a significant negative correlation between CD8$^+$ T-cell and macrophage percentages in human endarterectomy samples. This relationship could be indicative of a regulatory effect of CD8$^+$ T-cells on macrophages. In order to study this further, we depleted CD8$^+$ T-cells in the advanced stages of lesion development in LDLr$^{-/-}$ mice. The mice were fed a Western-type diet (WTD) for 10 weeks in order for lesions to establish and were kept on the WTD for another 6 weeks, during which they received injections of a monoclonal antibody to deplete CD8$^+$ T-cells, or an

isotype control antibody. We found that this treatment did not result in any differences in lesion size between the two groups. However, the absence of $CD8^+$ T-cells did result in an increase in macrophage content in the lesions, which is in agreement with the association we found between these two cell types in the human lesions. Moreover, depletion of $CD8^+$ T-cells resulted in a reduced lesion stability, as the collagen content of the lesions was decreased, whereas the necrotic areas were increased. When investigating the T-cell compartment within the lesions, we observed a striking increase in the percentage of inflammatory $CD4^+$ T helper 1 (Th1) cells associated with a decrease in percentage of Th2 cells upon CD8 depletion. Of note, this increase was only observed locally within the lesions and not at other sites in the body. Together, these results indicate that $CD8^+$ T-cells exert a protective effect in advanced atherosclerosis by reducing macrophage and Th1 cell content (Fig. 1). This is in agreement with previously published studies, demonstrating that antigen-specific $CD8^+$ cells can reduce atherosclerosis by mounting a cytolytic response against APCs [37].

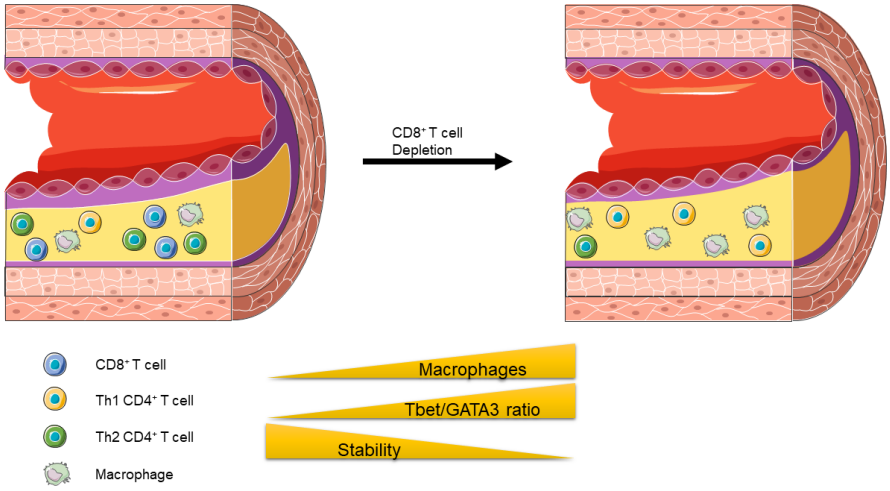

Figure 1: Schematic overview of the effects of $CD8^+$ T-cell depletion on the advanced stages of atherosclerosis. $CD8^+$ T-cell depletion increases plaque macrophage and Th1 cell content and reduces plaque stability. *From van Duijn et al. Cardiovasc Res. 2019 Mar 15;115(4):729-738.*

Indeed, we suggest a role for Fas-Fas ligand (FasL)-mediated cell death in establishing these atheroprotective effects. We found an increased expression of FasL on $CD8^+$ T-cells in the lesion microenvironment, which is known to induce apoptosis of activated $CD4^+$ T-cells [38], and Th1 cells specifically are known to have a high susceptibility to FasL-induced cell death [39, 40]. Moreover, lipid-loaded macrophages are vulnerable to Fas-FasL-mediated cell death as well [41]. Neutralizing the function of FasL in vivo using a monoclonal antibody resulted in a similar skewing of $CD4^+$ T-cell subsets in favor of Th1 cells, as well as an increased lesional macrophage content. Thus, the protective and lesion-stabilizing effect of $CD8^+$ T-cells in advanced atherosclerosis is at least in part mediated via FasL-induced apoptosis of Th1 cells and macrophages. This

protective effect of $CD8^+$ T-cells may be exploited therapeutically, by boosting $CD8^+$ T-cell function in the advanced stages of atherosclerosis, which should result in lesion stabilization.

3. $CD8^+$ T-cells modulate the immune environment locally within the lesion via several distinct mechanisms

In **Chapter 3** we observed that the function of $CD8^+$ T-cells within the lesion microenvironment differs from that in the secondary lymphoid organs. As the atherosclerotic microenvironment contains many lipid-derived and inflammatory stimuli, this may affect the $CD8^+$ T-cell phenotype specifically at this site. In **Chapter 4** we set out to investigate how the lesion microenvironment affects $CD8^+$ T-cell function. We demonstrated that $CD8^+$ T-cells derived from aortic lesions of $apoE^{-/-}$ mice show a dysfunctional phenotype, characterized by impaired cytokine production, when compared to their counterparts in the spleen. This phenotype was associated with an increased expression of the ectonucleotidase CD39. This enzyme is involved in the conversion of extracellular ATP, a danger-associated molecular pattern that has been linked to atherosclerosis development [42], into the immunomodulatory compound adenosine [43]. Adenosine produced by $CD8^+CD39^+$ T-cells has previously been shown to reduce cytokine production by other T-cells [44]. Indeed, we showed that pharmacological inhibition of CD39 is able to partly reverse the dysfunctional $CD8^+$ T-cell phenotype that was observed in the lesions of the $apoE^{-/-}$ mice. Mechanistically, we showed that TCR signaling induces expression of CD39 in the lesions. Transplantation of OT.1 bone marrow (which gives rise to $CD8^+$ T-cells unable to recognize any atherosclerosis-specific antigen) into $LDLr^{-/-}$ recipient mice resulted in a marked reduction in CD39-expression on lesion-derived $CD8^+$ T-cells compared to transfer of wild-type bone marrow. These results indicate that it is the antigen-specific stimulation of $CD8^+$ T-cells in the lesion environment that drives the unique phenotype observed at this location (Fig. 2). In agreement with this, recent work by others has shown that TCR activation induces reactive oxygen species that activate signaling cascades resulting in increased CD39 expression [44], and that TCR signaling specifically drives CD39 expression on $CD8^+$ T-cells in the tumor microenvironment as well [45, 46]. Importantly, we confirmed that in human atherosclerosis there is also a strong microenvironment-specific upregulation of CD39 on $CD8^+$ T-cells in the plaques compared to matched blood samples. Thus, the results we observed in our mouse models may be translated to a clinical setting, and boosting $CD39^+CD8^+$ T-cell function could be an interesting approach for the treatment of atherosclerosis.

In **Chapter 5**, we revisited lesion-localized $CD8^+$ T-cell responses, focusing on determining which $CD8^+$ T-cell subsets are present within the plaque microenvironment. Upon activation of $CD8^+$ T-cells, cytokines released by APCs can drive the differentiation of the $CD8^+$ T-cells into various subsets, which are characterized by distinct cytokine release profiles [47]. We observed that in the lesion microenvironment, there is a reduction in the content of IFN-γ-producing Tc1 cells, but a large increase in the percentage of Tc17 cells. To investigate how these cells affect atherosclerosis develop-

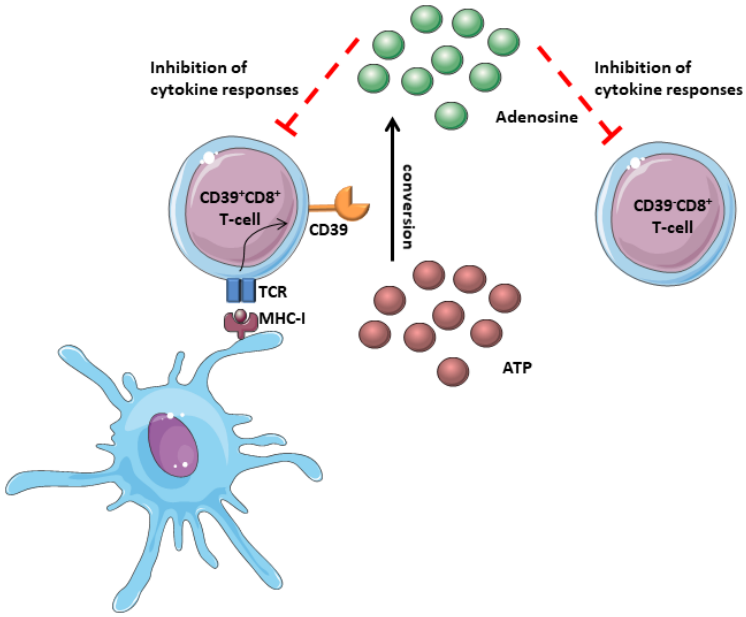

Figure 2: Schematic overview of the lesion-localized induction of CD39 on CD8$^+$ T-cells, and how this in turn affects the inflammatory status. TCR signaling induces CD39 expression on CD8$^+$ T-cells. The CD39$^+$CD8$^+$ T-cells convert pro-inflammatory ATP into anti-inflammatory adenosine, which in turn is able to reduce cytokine production by different CD8$^+$ T-cell subsets. *From van Duijn et al. Atherosclerosis. 2019 Jun;285:71-78*

ment, we performed an adoptive transfer of Tc17 cells or undifferentiated Tc0 cells into CD8-deficient LDLr$^{-/-}$ mice. Whereas Tc0 cells differentiated into a Tc1 phenotype upon injection, as evident from their increased IFN-γ production and T-bet expression, Tc17 cells demonstrated lower levels of IFN-γ production compared to the Tc0 cells and retained their ability to produce IL-17A. This shows that both of these subsets show plasticity in vivo in response to the inflammatory stimuli present in atherosclerotic mice [37, 48]. Adoptive transfer of Tc17 cells resulted in smaller atherosclerotic lesion size compared to the transfer of Tc0 cells. Moreover, there was a decrease in plaque macrophage content as well as a reduction in inflammatory CD4$^+$ Th1 cells in the aortas of the Tc17-treated mice (Fig. 3). As the Tc17 cells produced more IL-17 but fewer IFN-γ compared to the Tc0 cells, the atheroprotective function of Tc17 cells can be ascribed to either or both of these phenotypical differences. Nonetheless, these findings show that different Tc subsets are present in atherosclerotic lesions, and suggest that skewing the CD8$^+$ T-cell phenotype towards a less atherogenic subset, such as the Tc17 subset, may provide a novel therapeutic avenue for the treatment of atherosclerosis.

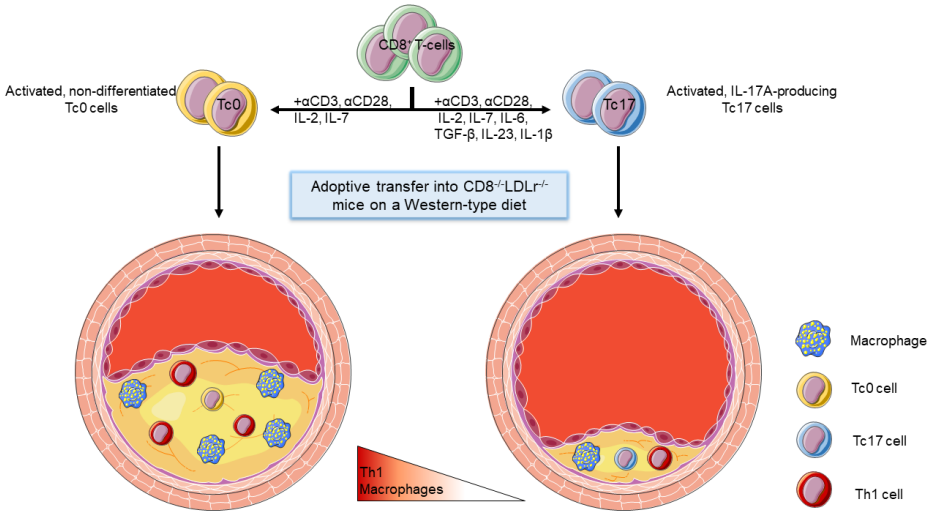

Figure 3: Schematic overview of how the adoptive transfer of Tc0 and Tc17 cells affects atherosclerosis development in CD8$^{-/-}$LDLr$^{-/-}$ mice. CD8^{+} T-cells were polarized towards a Tc0 or Tc17 phenotype and injected once weekly into CD8$^{-/-}$LDLr$^{-/-}$ mice fed a WTD for 6 weeks. Transfer of Tc17 cells resulted in smaller lesions associated with a reduced Th1 cell and macrophage content. *From van Duijn et al. Tc17 CD8^{+} T-cells accumulate in murine atherosclerotic lesions and modulate local inflammatory responses (submitted manuscript).*

4. CD8^{+} T-cell directed vaccination in atherosclerosis

As we determined that CD8^{+} T-cells can exert anti-atherogenic functions under certain conditions, we looked into ways in which we could harness the atheroprotective functions of CD8^{+} T-cells, while inhibiting their pro-inflammatory functions, as a potential treatment strategy for atherosclerosis. An interesting strategy to induce antigen-specific CD8^{+} T-cell responses is via vaccination. In **Chapter 6**, we reviewed how to design a particle-based vaccine in order to skew the immune response towards the desired direction. As pathogens have different dimensions and shapes, they stimulate the immune system in a specific way, inducing different immunological effects. Similarly, in this review, we focused on how the physicochemical characteristics of size, shape, and rigidity of the particulate antigen delivery system affects the biodistribution, cellular uptake, antigen presentation and the resulting immune response in murine models. Regarding the induction of CD8^{+} T-cell responses, we reviewed studies reporting that small particles in the nano-size range are easily transported from the site of injection to the lymphatic system [49]. Moreover, we discussed that small nanoparticles are taken up efficiently by APCs and localize rapidly into late endosomes [50], which subsequently fuse with the lysosomes from where the nanoparticles escape via the phagosome-to-cytosol pathway, resulting in higher MHC class I presentation [51]. Furthermore, we reviewed studies revealing that smaller sized nanoparticles are more effective than larger particles in inducing CD8^{+} T-cell responses, and this effect is most pronounced when these small particles are (nearly) spherical, probably due to

enhanced uptake of spherical particles [52, 53].

Using the knowledge we obtained regarding particulate vaccine design, we set out to induce atheroprotective $CD8^+$ T-cell responses via liposomal vaccination in **Chapter 7**. Liposomes are spherical vesicles composed of one or multiple bilayers of phospholipids which enclose an aquatic core. Liposomes are an efficient delivery system for antigens, as they enhance antigen uptake by APCs and initiate immune activation within these cells. Positively charged (cationic) liposomes below 200 nm in size were previously shown to induce strong antigen-specific $CD8^+$ T-cell responses in a cancer mouse model [54]. However, we were unable to induce antigen-specific $CD8^+$ T-cell responses in $LDLr^{-/-}$ mice by vaccinating against the ApoB100-derived peptide QSFDLSVK using cationic DOPC:DOTAP liposomes. Consequently, no effects of this treatment on atherosclerosis development were observed. This failure to induce immunogenicity was not due to the formulation, as we showed that vaccination using the exact same liposomes encapsulating the model peptide SIINFEKL was able to induce antigen-specific $CD8^+$ T-cell responses in $LDLr^{-/-}$ mice. Moreover, using dendritic cell vaccination in which the QSFDLSVK peptide was loaded directly onto the DCs, we were able to induce antigen-specific $CD8^+$ T-cell responses. Thus, immune responses can be directed against this peptide sequence in $LDLr^{-/-}$ mice. Most likely, there were issues with the stability of the peptide during the cross-presentation process in vivo, by which the antigen comes into contact with the acidic environment and proteases in the lysosomes before it gains access to the cytosol. As we have shown compelling data in this thesis supporting a protective role for $CD8^+$ T-cells via antigen-specific mechanisms, we believe further exploration into activating these cells could be of great value for atherosclerosis research.

5. Perspectives

In recent years, great progress has been made with regard to the research into immunotherapy of atherosclerosis. The aforementioned CANTOS trial [14] has already shown that targeting the inflammatory component of atherosclerosis can reduce the number of cardiovascular events, although adverse effects occur due to the general immune suppression. As $CD8^+$ T-cells are a part of the adaptive immune response, and therefore function in an antigen-specific manner, they could prove to be interesting tools for targeting the immune system in atherosclerosis in a more specific manner, reducing the occurrence of adverse effects such as the ones observed in the CANTOS trial. In this thesis, the contribution of $CD8^+$ T-cells to the pathogenesis of atherosclerosis has been studied, and several mechanisms through which $CD8^+$ T-cells exert protective functions have been identified. We hope these findings will prove to be useful starting points for developing therapeutic interventions in atherosclerosis.

In **Chapter 3**, we have shown a protective function for $CD8^+$ T-cells that was at least in part mediated via the increased expression of FasL on these cells. Activation-induced cell death, such as that induced by Fas/FasL signaling, is one of the main mechanisms used by the immune system to prevent autoimmunity, through controlling T-cell re-

sponses [55]. In mouse models, immunotherapies using genetic modulation of FasL were shown to reduce autoimmune diseases [56, 57], providing proof-of-concept for targeting the Fas/FasL-axis in atherosclerosis. Unfortunately, the administration of agonistic Fas antibodies or recombinant FasL results in severe hepatotoxicity and even death of animal models [58], eliminating the use of this strategy for the treatment of atherosclerosis. Alternatively, bispecific antibodies consisting of an agonistic Fas antibody hybridized to a second antibody directed against a different target antigen on the same cell were shown to be effective in inducing apoptosis of the target cells, while reducing non-specific adverse effects [59]. Potentially, this strategy could be employed to eliminate the atherogenic Th1 cells from the lesions. Alternatively, FasL-expressing $CD8^+$ T-cells could be employed as a therapy. Adoptive transfer of T-cells retrovirally transduced to overexpress FasL resulted in effective killing of prostate cancer cells in mice [60], suggesting it could be interesting to transduce patient-derived $CD8^+$ T-cells in a similar fashion ex vivo and subsequently transfer them back. It must be noted that none of the methods discussed above target the immune system in an antigen-specific manner, and thus a risk of generalized immune suppression remains a concern with these methods. In order to avoid this, one could consider the use of chimeric antigen receptor (CAR) T-cell therapy. These cells are constructed from T-cells isolated from the patient, which are genetically modified to express a CAR construct, which allows a cell to bind a certain target cell surface antigen via a single-chain variable fragment domain. This domain is linked to intracellular signaling molecules that are usually associated with T-cell receptor signaling, which induce T-cell activation upon binding of the antigen. Thus, a CAR T-cell can recognize antigens irrespective of MHC-I presentation but instead needs to bind a structure expressed on the target cell membrane. Upon recognition of this structure, CAR T-cells mediate their cytotoxic effect via the release of the cytotoxic perforin and granzymes, the release of cytokines, and via the Fas-FasL axis [61]. Thus, employing these T-cells in atherosclerosis would enable FasL-mediated killing of target cells in an antigen-specific fashion. For instance, once could design CAR T-cells that target scavenger receptor A-I, which is overexpressed on plaque macrophages, in order to kill these pro-atherogenic cells [62].

In **Chapter 4**, we describe that lesion-localized $CD8^+$ T-cells express high levels of CD39, which results in local modulation of inflammatory responses. As we and others have shown that TCR signaling is required to induce CD39 expression [44], it could be of interest to increase the number of CD39-expressing immunomodulatory $CD8^+$ T-cells via TCR signaling. Vaccination would provide an excellent method to upregulate CD39 expression, as this specifically activates $CD8^+$ T-cells in an antigen-specific manner. In **Chapter 7** we set out to test this hypothesis in an atherosclerotic mouse model. However, our liposomal vaccination method did not induce any antigen-specific $CD8^+$ T-cell responses toward the ApoB100-derived peptide. Therefore, we will have to be improve our strategy, using a more effective combination of peptide antigen and delivery system that is able to induce strong immune responses. It could be of interest to look into what antigens are naturally presented upon MHC-I molecules within the atherosclerotic lesions in murine models and human patients by the use of mass spectrometry [63]. This will provide an overview of what peptide fragments of which proteins are presented to $CD8^+$ T-cells in an atherosclerotic context and may thus lead

to the identification of a relevant antigen to boost atheroprotective $CD8^+$ T-cell functions in both preclinical and clinical studies. Once a suitable antigen is identified, it is important to assess if vaccination can indeed boost CD39- and FasL-expression levels on $CD8^+$ T-cells, reduce inflammatory $CD4^+$ T-cell responses, and induce cytolytic responses towards APCs. If this is found to be the case, there remains a need for studies exploring the potential of vaccination to treat atherosclerosis in humans, as most studies on vaccination in this disease have been performed in murine models [64]. Humans express different variants of the MHC-I molecules than mice, termed human leukocyte antigens (HLA), which have different binding affinities compared to their murine counterparts. Transgenic mouse lines that express HLA class I molecules have been developed [65], which may prove useful for investigating the translational value of a vaccination strategy. Finally, as we observed the increase in CD39 only in the lesional microenvironment, the effect of inducing CD39-expressing cells systemically will have to be studied. Possibly, most of the antigen-specific cells home towards the atherosclerotic sites, as this is where their cognate antigen is expressed, but one must be cautious of potential adverse effects.

As shown in **Chapter 5**, the skewing of $CD8^+$ T-cells towards different Tc subsets can affect the development of atherosclerotic lesions in a murine model. We report a protective effect of IL-17A-producing Tc17 cells compared to IFN-γ-producing Tc1 cells. Of note, treatment with a blocking antibody against IL-17A in psoriatic arthritis patients resulted in the occurrence of six major adverse cardiovascular events in the treated group, whereas no such events occurred in placebo-controlled patients [66]. Similarly, trials testing anti-IL-17A antibodies in ankylosing spondylitis resulted in three major adverse cardiovascular events in the treated group versus none in the controls [67]. Although these outcomes were not statistically significant, they could indicate that blocking the function of IL-17 may prove detrimental for the prevention of cardiovascular events, agreeing with the protective role we described for Tc17 cells. Conversely, administration of IL-17A may be beneficial against atherosclerosis development, although systemic administration may result in unwanted adverse effects. Therefore, adoptive transfer of IL-17A-producing $CD8^+$ T-cells, specifically targeted to the atherosclerotic lesions, may be preferable. Specific accumulation in the lesion may be enhanced by using $CD8^+$ T-cells that recognize antigens presented in the plaque. For instance, in the field of cancer research, adoptive transfer of ex vivo activated tumor-reactive $CD8^+$ T-cells skewed towards a Tc1 or Tc17 phenotype reduced melanoma growth [68]. Furthermore, adoptive transfer of antigen-specific OT.1 $CD8^+$ T-cells skewed towards the Tc17 phenotype controlled tumor-growth in early and late-stage melanoma in OVA tumor-bearing mice [69]. These experimental studies demonstrate the potential of combining adoptive T-cell transfer therapy with the skewing of those T-cells towards the desired phenotype ex vivo. Interestingly, ex vivo stimulation of cells under Tc17-polarizing conditions with a small-molecule RORγ-agonist was shown to potentiate the anti-tumor activity of both CAR-expressing human T-cells and tumor-specific $CD8^+$ T-cells [70], demonstrating that this approach could work in a clinical setting as well. Therefore, we suggest it is of interest to look into adoptive transfer of $CD8^+$ T-cells skewed towards the Tc17 phenotype ex vivo for the treatment of atherosclerosis, which allows for specific delivery of IL-17 to the atherosclerotic lesions.

Collectively, this research suggests there is potential to target $CD8^+$ T-cells in order to modulate the immune response in atherosclerosis. However, in doing so, one must be very careful to induce only the atheroprotective functions of the $CD8^+$ T-cells and monitor if no adverse effects are induced simultaneously. Moreover, much of the data demonstrating a protective role for $CD8^+$ T-cells has been collected in murine models, although we show that at least some of this can be translated to human atherosclerosis as well. Before an effective $CD8^+$ T-cell-based therapy can be developed, more research is required to confirm the atheroprotective function of this subset in human atherosclerotic lesions.

6. Conclusion

In summary, the research described in this thesis has shed new light on the phenotype and function of $CD8^+$ T-cells in atherosclerosis, demonstrating lesion-localized atheroprotective functions. This implies that antigen-specific stimulation of $CD8^+$ T-cell responses may have therapeutic potential. Great progress is currently being made towards developing antigen-specific T-cell therapies in numerous diseases. Moreover, immune-based therapies in general currently receive increasing attention for the treatment of atherosclerosis. Therefore, it is not a question of *if*, but rather of *when* effective immune-based treatment of cardiovascular diseases will be within our reach.

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