Novel immune cell-based therapies for atherosclerosis

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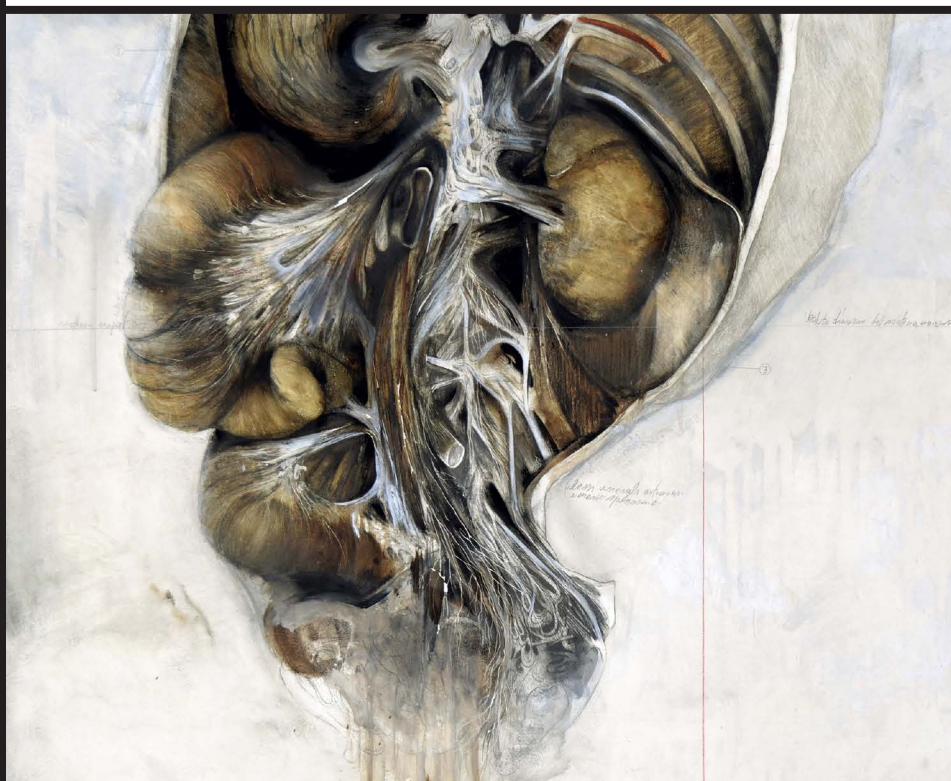
General Discussion and Perspectives



Background

Atherosclerosis is the main underlying cause of cardiovascular disease and mainly affects medium to large arteries. The disease develops due to dyslipidemia and inflammation, resulting in both a massive lipid accumulation and a chronic pro-inflammatory response in the vessel wall. Current treatment is primarily based on lowering blood cholesterol levels, one of the main risk factors for atherosclerosis. Statins, which were first marketed for their lipid lowering properties in the late 1980s, inhibit 3-hydroxy-3-methyl-glutaryl (HMG)-CoA reductase, which is the rate-limiting enzyme in cholesterol synthesis, but have also recently been suggested to have anti-inflammatory properties[1]. Nonetheless, statins can only lower cardiovascular risk by 25-30% and patients with coronary syndromes have a higher than 20% chance of a recurrent event, despite optimal treatment[2]. This emphasizes the urgent need for the development of new therapeutic strategies.

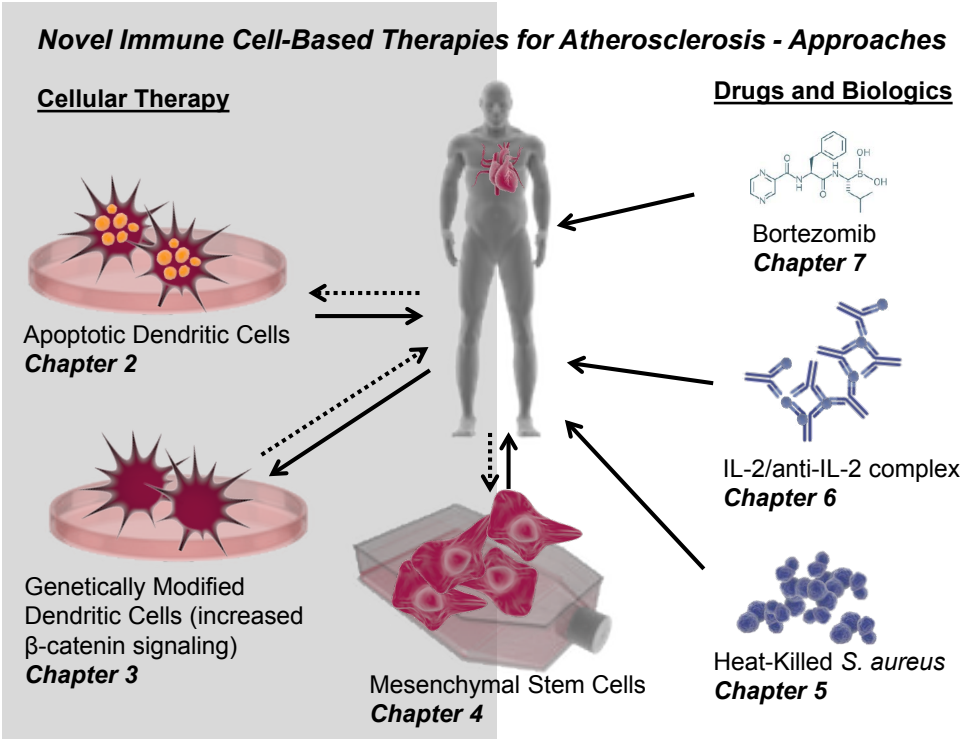


Figure 1. Approaches taken in this thesis to establish a novel immune cell-based therapy for atherosclerosis. Cellular therapy and drugs/biologics to modify immune cells were evaluated. Apoptotic DCs (chapter 2) and DCs with a β-catenin stabilization (chapter 3), as well as MSCs (chapter 4) were used as cellular therapy. MSCs were also used to indirectly modify immune cell function, thus, representing an intermediate approach. For indirect modulation of immune cells, we administered heat-killed *S. aureus* (chapter 5), IL-2/anti-IL-2 complexes (chapter 6), and the proteasome inhibitor Bortezomib (chapter 7).

This Thesis

The goal of this thesis was to establish novel immune cell-based therapies for atherosclerosis. Two approaches were used: **(1)** direct cellular therapy or **(2)** use of drugs and biologics to modify immune cell function in vivo to reduce atherosclerosis in

low-density lipoprotein receptor-deficient ($LDLr^{-/-}$) mice, which develop atherosclerosis on a high-fat (Western-type, WTD) diet (Figure 1). While our therapeutic strategies were specifically aimed to reduce inflammation, we observed in two studies a beneficial effect on lipid homeostasis.

(1) Cellular Therapy: Direct Delivery of (Modified) Immune Cells to Treat Atherosclerosis

The goal of an anti-inflammatory treatment for atherosclerosis is to modulate ongoing inflammatory responses of both the innate and adaptive arm. Dendritic cells (DCs) are the most potent antigen-presenting cells and bridge innate and adaptive immune responses. We hypothesized that modulation of DCs towards a tolerogenic phenotype, i.e. producing anti-inflammatory mediators and inducing regulatory T cell (Treg) responses can beneficially affect atherosclerosis. We addressed this in two ways: (1) by administration of apoptotic DCs, which results in tolerogenic DC responses, and (2) by increasing β-catenin signaling in DCs to directly induce a tolerogenic phenotype.

In **chapter 2**, we investigated the potential of an adoptive transfer of oxidized LDL (oxLDL)-induced apoptotic DCs in both early and advanced atherosclerosis. It has been established that clearance of apoptotic cells (efferocytosis) in advanced atherosclerotic lesions is impaired[3] and earlier studies by our lab and others have shown that blockade of efferocytosis worsens atherosclerotic disease burden in experimental models for atherosclerosis[4,5]. Here we provide first evidence that clearance of oxLDL-induced apoptotic DCs has the potential to induce atheroprotective responses. We show that administration of oxLDL-induced apoptotic DCs results in induction of tolerogenic DCs, increased numbers of regulatory T cells, and reduced inflammatory monocyte responses.

After adoptive transfer of apoptotic DCs, either before lesion initiation or after established lesions had developed, we found that apoptotic DCs were mainly cleared by DCs and macrophages in the marginal zone of the spleen, inducing a tolerogenic phenotype and enabling an induction of Treg responses. Additionally, we found that overall (inflammatory) monocyte numbers were reduced, a previously unknown effect of apoptotic cell treatment. Moreover, in already established lesions, which resemble a more clinical setting as most patients enter the clinic with evident atherosclerosis, our treatment was able to increase lesion stability. Our study not only indicates the potential of such a therapeutic approach for the clinic, but also emphasizes the importance of research into the emigration potential of DCs from lesions. This is further reinforced by an earlier study from our laboratory showing that the adoptive transfer of oxLDL-pulsed DCs reduces initial atherosclerosis[6]. If DCs, which clear oxLDL or oxLDL-induced apoptotic DCs/macrophages (both containing atherosclerosis-relevant antigens), can be induced to emigrate early on from lesions, they might in fact contribute to inhibition of lesion development and stabilization.

Interestingly, at the same time as we published our results, Hosseini *et al.* found that *intraperitoneal* administration of apoptotic thymocytes also reduced atherosclerotic lesion development in $ApoE^{-/-}$ mice[7]. Similar to our study, mice show reduced numbers of macrophages and T cells in atherosclerotic lesions. However, it will

be interesting to determine why we mainly observed effects on monocyte responses and Hosseini *et al.* mainly found effects on B cell responses, specifically an induction of atheroprotective B1a cells producing IgM[7].

Another interesting aspect of our study was that control apoptotic DCs, which were not loaded with oxLDL, did reduce inflammatory cytokine responses, but did not induce tolerogenic DC phenotypes when taken up by bone marrow-derived or splenic DCs. This is in line with the study by Hosseini *et al.* who did not find any effect on Treg responses, as their apoptotic cells did not contain oxLDL[7]. We found that only DCs exposed to oxLDL-induced apoptotic DCs upregulated the expression of PD-L1, IL-10, ABCA1 and ABCG1, indicating that liver X receptor (LXR) signaling was increased. Indeed exposure to apoptotic cells and cholesterol both induce LXR activation[8–10]. In fact, LXR activation of DCs has been shown to result in a tolerogenic DC phenotype[11]. We therefore speculate that while oxLDL-induced apoptotic DCs might indeed transfer an atherosclerosis-relevant antigen in the context of an anti-inflammatory setting (efferocytosis), excess cholesterol present in apoptotic cells might contribute to the additional benefit we observe using oxLDL-induced apoptotic DCs.

It has also been suggested that phosphatidylserine (PS)-containing liposomes, which are thought to mimic apoptotic cells, can modulate inflammatory responses. PS is needed for apoptotic cell recognition by DCs and macrophages and inhibits DC maturation[12–14]. Indeed, *intravenous* administration of PS-containing liposomes induces anti-inflammatory TGF-β and IL-10 responses by macrophages, which improves infarct repair after myocardial infarction (MI)[15]. This approach would enable an easier translation to the clinic as liposomes could be generally produced. Indeed Hosseini *et al.* found that PS liposomes could mimic apoptotic cells and attenuate atherosclerosis in ApoE$^{-/-}$ mice[7]. However, in a preliminary study we did not find any effect of a lower dose of PS-containing liposomes on atherosclerosis development in LDLr$^{-/-}$ mice.

In another approach to induce tolerogenic DCs, we assessed whether increased β-catenin signaling in DCs could have a beneficial effect on atherosclerotic lesion development. In **chapter 3**, we made use of CD11c-βcat^{EX3} mice, which have constitutively active β-catenin signaling in all CD11c^{+} cells. β-catenin signaling in DCs was previously suggested to be involved in promoting a tolerogenic phenotype in DCs[16–18]. Indeed, upon bone marrow transplantation of CD11c-βcat^{EX3} bone marrow into LDLr$^{-/-}$ mice, we found a significant increase in Tregs, which reduced aortic root lesions by 26%. These lesions had significantly less necrotic areas and showed a trend towards more collagen. Similarly, an adoptive transfer of CD11c-βcat^{EX3} DCs into LDLr$^{-/-}$ mice decreased atherosclerotic lesion development by 21%. However, we used CD11c as a promoter in this study and it has to be acknowledged that CD11c is not an exclusive marker for DCs[19]. Monocytes and macrophages can also express CD11c, especially under hypercholesterolemic conditions[19,20]. Nonetheless, also in macrophages Wnt3a signaling, which induces β-catenin, was found to result in an anti-inflammatory phenotype[21] and this may account for some of the beneficial responses we observed after bone marrow transplantation. In the future it will be interesting to determine the effect of an adoptive transfer of DCs with a β-catenin stabilization in

advanced atherosclerosis, possibly with an increased treatment regimen e.g. three doses about two weeks apart as DCs have a half-life up to a week[22] and a similar regimen is advised for Provenge, a DC-based therapy for prostate cancer[23].

In recent years interest in the potential and benefit of stem cell therapies has increased and stem cells have frequently been described to "cure" multiple diseases. Indeed, tremendous advances have been made in stem cell research and in stem cell use in regenerative medicine. Mesenchymal stem cells (MSCs) specifically have been used for e.g. bone regeneration[24], neuroregeneration[25] and myocardial regeneration[26]. Recently, the immunoregulatory properties of MSCs have been acknowledged, since MSCs have a beneficial effect on inflammatory diseases as established in experimental mouse models for experimental autoimmune encephalomyelitis, collagen-induced arthritis, and type 2 diabetes[27–30] and in clinical trials for graft-versus-host disease and multiple sclerosis[31,32]. In **chapter 4**, we therefore adoptively transferred mouse MSCs into LDLr$^{-/-}$ mice on a WTD and found that MSCs reduced circulating CCL2 levels, monocytes and effector CD4^{+} and CD8^{+} T cells. Additionally, we found a striking effect on lipid metabolism: MSCs reduced total cholesterol levels by significantly decreasing VLDL levels as a result of a decreased *de novo* lipid synthesis. Overall, MSC therapy resulted in a 33% decreased atherosclerotic lesion development.

The effect of MSC therapy on cholesterol metabolism is novel, but since the effect was only seen four to five weeks after injections and atherosclerosis induction, we speculate that the effect is an indirect result of reduced inflammation. For example IL-10 has been found to decrease VLDL synthesis[33], while TNF-α increases VLDL synthesis[34], indicating that modulation of cytokine responses by MSCs could already reduce dyslipidemia. Because MSCs have been investigated for their role in cardiac repair after MI, it would be interesting to also establish effects on cholesterol levels in in these patients. Interestingly, in a small 18 patient cohort, it was shown that intracoronary stem cell infusion following MI reduced plaque burden in the coronary tree four years after treatment[35], indicating MSC treatment can be beneficial for atherosclerotic patients.

Two recent studies have provided evidence that MSCs can ameliorate atherosclerosis. Lin *et al.* demonstrate that MSC therapy improved vasodilation, by increasing eNOS expression in endothelial cells, and thereby reduced atherosclerotic lesions in the aorta of ApoE$^{-/-}$ mice[36]. Another study by Wang *et al.* found that MSC reduced atherosclerotic lesions in the aortic root in ApoE$^{-/-}$ mice, by inducing Tregs[37]. While the first study reported no effect on serum cholesterol levels[36], the second study does not mention total serum cholesterol levels. Lin *et al.* adoptively transferred human MSCs into ApoE$^{-/-}$ mice, harboring the risk of xenograft rejection. Wang *et al.* used MSCs derived from ApoE$^{-/-}$ mice, while we used MSCs from a C57BL/6 background. Increased cholesterol in stem cells of ApoE$^{-/-}$ mice has been found to increase proliferation of hematopoietic stem and progenitor cells[38], suggesting that disturbed cholesterol homeostasis of ApoE$^{-/-}$ MSCs could result in the differences to wild type MSCs. Future studies will have to address which type of MSCs, when and how often they should be administered.

(2) Drugs and biologics to modulate immune cell function in vivo

In addition to cellular therapy, drugs and biologics can be used to modulate immune cell function or to induce a specific immune cell subset in vivo.

In a first approach we made use of the fact that the immune system has an intrinsic control mechanism to reduce inflammatory responses when a pathogen is eliminated to ensure a return to tissue homeostasis. Toll-like receptors (TLRs), which recognize pathogens, have been found to in addition to initial inflammatory responses, result in anti-inflammatory IL-10 production[39]. Several studies have suggested that TLR2 activation specifically induces IL-10 responses[39–41]. A fundamental difference appears to exist between different types of antigen presenting cells in their capacity to produce IL-10: macrophages produce high levels of IL-10 in response to TLR2 signaling, while DCs produce much lower amounts of IL-10[40,42]. In **chapter 5**, we show that *intraperitoneal* injections of heat-killed *Staphylococcus aureus* (HK-SA) induce a potent anti-inflammatory IL-10 response, which is capable of reducing inflammation and thereby reduces atherosclerotic lesion development in the aortic root by 34%. We demonstrate that IL-10 responses are crucially dependent on TLR2/PI3K signaling and that this induces an immunoregulatory M2b phenotype in macrophages. The strong IL-10 production reduces the expression of adhesion molecules (VCAM-1 and ICAM-1) and CCL2 in the aorta. The amount of circulating inflammatory Ly-6C^{hi} monocytes and the amount of lesional macrophages is dramatically reduced. Additionally less Th1 and Th17 responses were observed and also lesional T cell numbers were reduced by HK-SA treatment. Here we demonstrate that TLR2/PI3K-dependent signaling can be exploited to induce anti-inflammatory IL-10 responses, which reduce monocyte/macrophage and T cell responses, as a treatment for atherosclerosis.

Several immunomodulatory strategies induce immunoregulatory/tolerogenic cells to reduce inflammation. One of the most investigated immune cells in the context of atherosclerosis is the Treg. In earlier chapters (chapter 2, 3, and 4) our treatment indirectly increased Tregs and this contributed to beneficial effects on atherosclerotic lesion development. However, in these studies we only achieved a modest increase (1.5-fold to 1.8-fold) and these generally did not last throughout the entire experiment. In **chapter 6**, we therefore assessed whether an immense and prolonged increase in Tregs, by administration of an IL-2/anti-IL2 complex, could reduce atherosclerotic lesion development and induce regression of pre-established lesions in parallel to a switch to a normal chow diet. This treatment resulted in an overall 10-fold induction of Tregs and increased splenic IL-10 responses. Treg expansion prevented atherosclerotic lesion development by 39%, suggesting that Tregs can potently inhibit initial atherosclerosis. However, while Tregs did not affect lesion size in established atherosclerosis, IL-2/anti-IL-2 complex treatment did increase lesional collagen content by 21%, indicating a more stable lesion phenotype. The latter is more clinically relevant as patients in the clinic will already have lesions and it suggests that a vast Treg expansion could stabilize such lesions.

Macrophages and DCs present atherosclerosis-relevant antigens in the context of cytokine production and can thereby modulate T cell responses. For presentation of antigens on major histocompatibility complex (MHC) I, antigen-processing by the

proteasome is required. Additionally the proteasome plays a central role in several inflammatory signaling pathways in atherosclerosis as it degrades key signaling/ inhibitory molecules, e.g. in TLR, TNF receptor and T cell receptor signaling pathways. However, also signaling proteins and transcription factors involved in lipid metabolism are degraded by the proteasome. We therefore in **chapter 7** sought to investigate if proteasomal inhibition by Bortezomib could both reduce inflammation and reduce dyslipidemia. Indeed, we found a significant reduction of monocyte/macrophage responses: reduced absolute (inflammatory) circulating monocytes and reduced macrophages in the aortic arch, aortic root and liver were observed. We also found reduced Th1 responses and increased Th2 responses. But more strikingly, we found dramatic effects on VLDL metabolism. The VLDL secretion rate was more than half reduced in mice treated with Bortezomib, which resulted from a significant decrease in *de novo* lipid synthesis and an increased bile acid secretion by the liver. Bortezomib consequently resulted in decreased inflammation, but also dramatically reduced hepatic steatosis, which culminated in a robust 58% reduction in atherosclerotic lesion size. Effects on monocytes were in line with a study by Wilck *et al.*, but they did not describe effects of Bortezomib on T cell subsets or lipid metabolism[43]. Interestingly, however, they do mention a reduction of the expression of HMG-CoA reductase in the aorta, but measurements in the liver were not performed. Likely the therapeutic dose for lipid lowering was not achieved in their study and it is therefore interesting to determine the minimal dose of Bortezomib to still affect lipid metabolism.

Considerations

In this thesis preclinical mouse models for atherosclerosis were used and it has to be noted that immune cell numbers largely differ from humans. Humans have about 30% lymphocytes and 60% neutrophils, while C57BL/6 mice have about 80% lymphocytes and 10% neutrophils[44]. Monocytes represent up to 15% of circulating leukocytes in humans and represent three subsets, while only up to 4% are found in mice with two subsets[45,46]. However, whether these differences in immune cell numbers translate into functional differences remains to be established. Additionally LDLr$^{-/-}$ and ApoE$^{-/-}$ mice already have increased total cholesterol levels on a regular chow diet: 175-225mg/dL and 400-500mg/dL, respectively. Upon WTD these levels can increase over 2000 mg/dL[47–49]. In contrast cholesterol levels in humans above 190 mg/dL are already considered as very high[50]. Atherosclerosis development in mice and men has one fundamental difference: lesion rupture and thrombosis are not observed in mice[51]. Furthermore, most treatment strategies are currently assessed by their effect on atherosclerotic lesion development in mouse models, while patients in the clinic mostly present with pre-established lesions. These differences have to be kept in mind when translating murine pre-clinical studies to human clinical trials, i.e. from "bench-to-bedside". For example the drug dose translation from animals to humans cannot simply be done by conversion according to body weight and specific formulas have been established and are recommended by the United States Food and Drug Administration (FDA)[52].

Despite these differences between mice and men, initial atherosclerosis development

is similar, making the mouse a relevant model system. Recently a large scale genomic correlation study additionally found that murine inflammatory responses mostly correlate with genomic responses in humans[53], suggesting that mouse models are good models for human inflammatory diseases. Indeed, a vast amount of novel insights into the atherosclerotic disease process stem from pre-clinical murine studies. Recently there have been efforts to "improve" mouse models further by generating more "humanized" mice with a human hematopoietic system to make mouse-human translations easier[54].

Perspectives

Immune cell therapies have been thoroughly investigated in cancer. Already in 1955, it has been described that adoptive transfer of cells from tumor-draining lymph nodes to a secondary host could confer immunity to the tumor[55]. Since then several advancements have been made to develop T cell therapies for cancer, which are currently successfully investigated for chemotherapy-resistant leukemia in clinical trials[56] and the first DC therapy for prostate cancer has been approved by the FDA[23]. In type 1 diabetes and rheumatoid arthritis clinical studies investigating the potential of tolerogenic DCs are still ongoing[57,58]. In renal transplantation 'the ONE study' (A Unified Approach to Evaluating Cellular Immunotherapy in Solid Organ Transplantation) was set up as a cooperative project to find and develop an immunoregulatory cell (product)-based therapy[59]. It will be interesting to follow the outcome of these comparative studies of different cell therapies. Additionally, MSCs have been investigated in clinical trials e.g. to enhance cardiac function after MI[60] and to reduce acute graft-versus-host disease[31]. Overall these initial clinical studies indicate that cell therapies are well-tolerated and lack side-effects. For MSCs it has been established in clinical trials that they can reduce inflammatory responses in some diseases, however, the outcome of clinical trials for DC therapy of inflammatory diseases is still anticipated.

In this thesis, we investigated cell therapies with apoptotic DCs, DCs with a β-catenin stabilization, and MSCs. The treatment with oxLDL-induced apoptotic DCs in **chapter 2** is translatable into the clinic. Both DCs and LDL can be isolated from patients and can therefore generate a patient-tailored therapy. For the treatment of multiple sclerosis a similar approach has been used: coupling of antigenic myelin peptides to apoptotic peripheral blood mononuclear cells derived from patients. Last year a phase I clinical trial showed this approach to be safe and to decrease antigen-specific T cells[61]. This trial provides first evidence in men that antigen-coupled apoptotic cells results in antigen-specific tolerance. In graft-versus-host disease, extracorporeal photopheresis has been approved by the FDA and is employed to reduce immune responses. Here patient-derived leukocytes are irradiated to induce apoptosis and are re-infused, which induces Tregs[62]. These studies provide evidence that our approach is not only promising and feasible, but should be well-tolerated by patients. Nonetheless, in both cases no effects on monocytes have been described and it would be worthwhile to investigate this. Treatment with apoptotic DCs should prove beneficial for atherosclerosis and unstable angina, but could also prove beneficial after MI, which results in a massive recruitment of monocytes and a worsening of

underlying atherosclerosis. Apoptotic cell treatment could reduce these monocyte responses and simultaneously stabilize lesions, preventing a recurrent event. However, treatment would have to be immediately upon MI and could therefore not include oxLDL loading or DC culture, so here the potential of extracorporeal photopheresis should be explored.

In atherosclerosis, vaccination with IL-10-treated ApoB100-pulsed (tolerogenic) DCs has been shown to reduce lesion development in the aorta by 70%, due to an inhibition of T cell responses[63]. In an opposite approach, our laboratory has shown that DC therapy with oxLDL-pulsed LPS-stimulated (mature) DCs also reduced carotid artery lesion development induced by collar placement by 85%. The reduced lesion was found to likely result from the induction of oxLDL-specific IgG antibodies. However, a similar study using malondialdehyde-modified (MDA) LDL-pulsed LPS-stimulated (mature) DCs found an aggravation of atherosclerosis[64]. The differences between these two studies were that one used wild type DCs and the other $ApoE^{-/-}$ DCs. It could therefore be interesting to determine whether the DC phenotype accounted for this difference as this will enable a better targeted modulation of DCs for atherosclerosis therapy. We therefore in **chapter 3** used tolerogenic DC therapy as this seemed a more straightforward approach to reduce inflammation. Indeed, we show that adoptive transfer of tolerogenic DCs can reduce atherosclerotic lesions providing further evidence that a tolerogenic DC-based therapy is a feasible approach to treat atherosclerosis. Moreover, we show that enhancing β-catenin signaling in transferred DCs could increase their therapeutic potential.

In addition to DC therapies, we describe in **chapter 4** that MSCs can significantly inhibit atherosclerotic lesion development. Because MSCs have been found to have enhanced immunoregulatory properties under inflammatory conditions[65,66], we can envision that a MSC therapy during ongoing atherosclerosis could have a more pronounced effect. Interestingly, a recent study found that MSCs can recruit myeloid-derived suppressor cells[67], which have very potent anti-inflammatory properties. It could therefore prove interesting to combine a MSC therapy with a myeloid-derived suppressor cell therapy to increase the amount of myeloid-derived suppressor cells that can be recruited to lesions.

A challenge for cellular therapies in general is that upon injection cells will be exposed to a different environment, which will affect them and unavoidable influence their phenotype. Therefore genetic engineering may be a promising approach for cell therapies in the future. More specifically, the direct genetic modification of immune cells, as e.g. stabilization of β-catenin, will enable the adoptive transfer of potent immunoregulators that execute targeted actions without undergoing differentiation or modulation in vivo. However, translating such a strategy to clinical practice will currently be difficult due to fear of genetic modifications in the general public. As an alternative, to avoid complications when administering cells, immune cell functions could be directly targeted. As the cells are already in their environment, modulation will occur after they have been exposed to other factors. This ensures that induced phenotypes are not reverted.

In **chapter 5**, we found that macrophages exposed to HK-SA produce a vast

amount of IL-10 and express other anti-inflammatory molecules, such as IDO, PD-L1, and CCL22. We found that TLR2/PI3K signaling is needed for this modulation, indicating that this signaling pathway can be exploited to induce anti-inflammatory responses. We thus challenge the current understanding that TLR2 and PI3K signaling are pro-atherogenic. It will be interesting to develop small molecules that can modulate TLR2 and PI3K signaling in a similar way to HK-SA and possibly specifically target these small molecules to lesional macrophages, as a therapy for atherosclerosis.

As previously mentioned a direct approach to induce tolerance would be to induce a significant expansion of Tregs, which we did in **chapter 6** by administration of an IL-2/anti-IL-2 complex. This approach could be translated in two ways to the clinic: either by direct administration of this complex to expand Tregs in vivo or by isolating T cells and expanding Tregs in vitro. In clinical trials, low dose subcutaneous administration of IL-2 (Proleukin®) was found to induce Tregs and resulted in clinical improvement of hepatitis C virus-induced vasculitis[68], graft-versus-host disease[69] and type 1 diabetes[70,71]. The potency of IL-2 was found to be increased in complexes with IL-2 monoclonal antibodies, which was found to significantly expand Tregs in mice and e.g. potently reduce experimental autoimmune encephalomyelitis, islet transplantation and asthma[72,73]. We have shown that these IL-2 complexes can also reduce atherosclerosis development and increase stability of advanced lesions in $LDLr^{-/-}$, which was later confirmed by another group in $ApoE^{-/-}$ mice[74]. Future studies will have to determine whether these IL-2 complexes are superior to regular IL-2 administration in patients and whether its administration is safe. IL-2 in a combination therapy with rapamycin has also been found to increase Tregs in patients with type 1 diabetes but was found to result in β-cell dysfunction[75]. The combination of IL-2 complexes with rapamycin could also be beneficial as has been found in an animal model of graft-versus-host disease[76].

Tregs can also be isolated from patients and then expanded in vitro before administration. This approach has been found to be safe and effective in clinical trials for graft-versus-host disease, transplantation, and autoimmunity[77]. Future studies will have to determine whether direct in vivo expansion or in vitro expansion and subsequent administration is more beneficial. An exciting challenge will also be the generation of antigen-specific Tregs for atherosclerosis therapy. Our laboratory has shown that antigen-specific Tregs can be generated by oral tolerance induction to oxLDL and HSP60[78,79]. A combination treatment to induce antigen-specific Tregs and then expand them seems most favorable.

Atherosclerosis is both determined by dyslipidemia and inflammation and we show in **chapter 7** that Bortezomib can reduce both underlying causes. As Bortezomib targets different enzymes in lipogenesis than statins, it is likely that combination therapies would be able to more dramatically reduce cholesterol levels. Moreover, Bortezomib could be effective in patients unresponsive to statin treatment. In addition, its anti-inflammatory effect could also compliment anti-inflammatory effects of statins, which have been for example found to induce eNOS in endothelium, to inhibit pro-inflammatory cytokine production by VSMCs, to inhibit B cell proliferation, to inhibit platelet function, and to decrease lesional T cells and macrophages[80]. Due to

their differential effects on lipid metabolism and inflammation, combination treatment with statins and Bortezomib should be greatly beneficial.

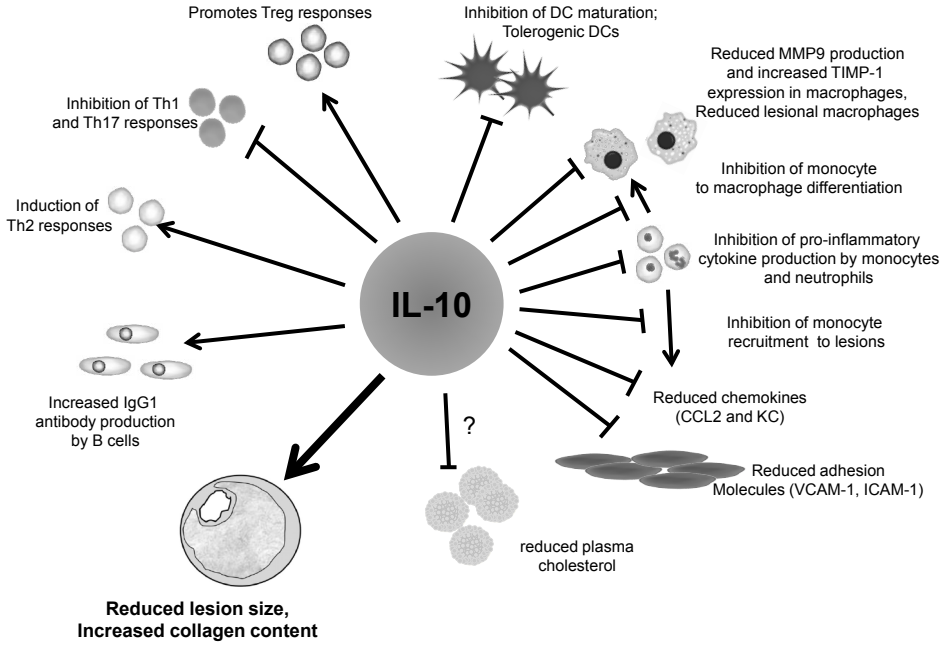


Figure 2. IL-10 affects multiple inflammatory responses in atherosclerosis and has been implicated in affecting serum cholesterol levels. Overall, IL-10 has been shown to contribute to atherosclerotic lesion size reduction and increased collagen content of lesion. Th, T helper; DC, dendritic cell; MMP, matrixmetalloproteinase; TIMP, tissue inhibitor of metalloproteinases; CCL2, chemokine (C-C motif) ligand 2; KC, keratinocyte chemoattractant; VCAM-1, vascular cell adhesion molecule 1; ICAM-1, intercellular adhesion molecule 1; IgG1, Immunoglobulin G1.

All of our studies induced IL-10 responses (chapters 2, 3, 4, 5, 6, and 7) and some induced Treg responses (chapter 2, 3, 4, 6), which also produce IL-10. SNPs in the IL-10 promoter region have been found to be associated with several risk factor for atherosclerosis and the pathobiology of atherosclerosis[81,82]. Indeed, IL-10 has been found to have extensive effects on atherosclerosis (Figure 2). Numerous studies have assessed the beneficial effects of IL-10 on atherosclerosis, such as adenoviral gene transfer[33,83,84], transgenic mice expressing IL-10 under the IL-2 promoter[85,86] or the CD68 promoter[87], and IL-10 deficient C57BL/6 mice[85,88] and ApoE$^{-/-}$ mice[89]. Interestingly, some studies found that IL-10 reduces cholesterol levels[33,83], while others found enhanced cholesterol[89], or no effects[84,85,88]. In general, IL-10 potently affects many inflammatory processes, for example it reduces expression of adhesion molecules on endothelial cells[85,90], reduces CCL2 levels[83], inhibits DC maturation[90], affects monocyte responses[90], reduces lesional macrophages[84], increases macrophage cholesterol uptake and clearance[87], modulates T cell responses[88,90], affects B cell responses[90] and increases lesional collagen content[88]. This makes IL-10 a potent anti-inflammatory cytokine.

It could well be argued that a large part of the responses we observe in our

studies is mediated by IL-10. However, the clinical development of recombinant human IL-10 (Tenovil) produced by Schering Plough was discontinued due to insufficient beneficial effects in Crohn's disease[91]. Additionally, cellular therapies or specific drugs/biologics offer the advantage of a targeted approach, i.e. defined cells will be modulated, which can migrate to/reside in lesions or draining lymph nodes to modulate specific responses. Cells can be engineered to perform more than one (specific) task and to adapt/respond to their environment. However, the most promising aspect of cellular therapy is the potential to induce antigen-specific tolerance. This would ensure a more targeted therapy and leave other immune responses, such as an ongoing infection, unaffected.

In the past, several other anti-inflammatory strategies for atherosclerosis have been explored. Just to name a few: subcutaneous vaccination with native LDL or antigenic peptides from LDL[92–96], intramuscular vaccination against IL-12[97], immunization against apoB100-specific TCRs[98], CD40L blockade[99,100] and OX40L blockade[101,102] have all been evaluated in preclinical animal models. Anti-inflammatory approaches are now starting to be validated in first clinical trials. Currently two large placebo-controlled clinical trials, the Cardiovascular Inflammation Reduction Trial (CIRT) and the Canakinumab Anti-Inflammatory Thrombosis Outcomes Study (CANTOS), are ongoing to establish whether lowering inflammation will reduce cardiovascular events. The CIRT evaluates the effect of methotrexate[103], which in low doses is anti-inflammatory, and the CANTOS evaluates the effect of inhibiting IL-1β[104]. In phase IIb of the CANTOS it was found that treatment with canakinumab (anti-human IL-1β monoclonal antibody) reduces inflammation in patients, as determined by reduced IL-6 and C-reactive protein, a marker for cardiovascular risk[105], indicating its potential. Both methotrexate and canakinumab are already approved by the FDA for other inflammatory diseases, e.g. juvenile idiopathic arthritis. Additionally other strategies have been validated in first clinical trials, such as a P-selectin antagonist (RO4905417)[106], 5-lipoxygenase inhibitor (Atreleuton) reducing leukotriene production[107], and an anti-CCR2 antibody (MLN1202)[108].

Furthermore, several anti-inflammatory therapies have been tested in other autoimmune diseases and could possibly be translated to atherosclerosis, e.g. abatacept and anti-TNF-α therapies. Abatacept (a fusion protein of IgG and CTLA-4) binds and blocks CD80/CD86 and thereby reduces T cell responses. It has been approved by the FDA for the treatment of rheumatoid arthritis and juvenile idiopathic arthritis; and has been indicated to reduce experimental atherosclerosis[109]. Additionally, anti-TNF-α therapies have been approved by the FDA for the treatment of rheumatoid arthritis and Crohn's disease, among others. In mice, TNF-α inhibition or deficiency has been described to either have no effect[110] or to reduce atherosclerosis[111,112]. In patients, a clinical trial of infliximab (monoclonal antibody against TNF-α), however, found that at high doses of 10 mg/kg TNF-α patients had a much higher risk of heart failure[113]. This clearly indicates that careful evaluation of therapies and their treatment regimens is needed when translating from mice to men

It will be interesting to follow the outcome of large clinical trials such as the CIRT and CANTOS as they will establish on a large scale for the first time whether

interfering with inflammatory pathways will reduce cardiovascular events. As not all patients enrolled in the CIRT and CANTOS trial are taking statins[103,104,114], it could be interesting to determine if combination with statin use is more beneficial. Likely, as atherosclerosis is determined by dyslipidemia and inflammation, the future of cardiovascular treatment strategies will lie in a combination treatment for reducing lipids and inflammation. When comparing all approaches we took in this thesis, it becomes evident that strategies that only affect inflammation result in at most a 40% reduction of lesion sizes, while only when both inflammation and lipid metabolism is dramatically reduced by Bortezomib a more significant lesion reduction of up to 58% is possible (Table 1). Therefore, the future of cardiovascular treatment strategies will lie in reducing both inflammation and dyslipidemia, either by administering drugs potently affecting both (such as bortezomib), or by combination therapies.

In summary, our studies confirm that immune cell therapies show great potential for the treatment of atherosclerosis. However, several challenges in translating these studies into the clinic remain. The journey towards immune cell-based therapies for atherosclerosis, as well as other diseases, will unquestionably be a very exciting one.

Chapter	Therapy	Results	% lesion reduction	% collagen increase
2	**oxLDL-induced apoptotic DCs**	- Reduced monocyte responses - Tolerogenic DCs - Increased Tregs	**28%** (D) **40%** (P)	n/a **45%**
3	**β-catenin stabilization in DCs**	- Tolerogenic DCs - Increased Tregs	**21%** (D) **26%** (BMT)	n/a **25%**
4	**MSCs**	- Reduced T cell responses - Reduced VLDL	**33%** (D)	n/a
5	**HK-SA**	- Reduced monocyte responses - Reduced T cell responses - Increased IL-10 producing M2b-like macrophages	**34%** (D)	n/a
6	**IL-2/anti-IL-2 complex**	- Increased Tregs	**39%** (D) **0%** (R)	n/a **21%**
7	**Bortezomib**	- Reduced monocyte responses - Reduced T cell responses - Reduced VLDL	**58%** (D)	n/a

Table 1. Overview of results of studies presented in this thesis. All studies affected several inflammatory responses; only studies of chapters 4 and 7 (indicated in dark grey) found effects on lipid metabolism. BMT, Bone Marrow Transplantation; D, Development of atherosclerosis; P, Progression of atherosclerosis; R, Regression of atherosclerosis, n/a: not affected likely due to initial stages of atherosclerosis.

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