Regulation of immune responses in atherosclerosis

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Chapter 10

Summary and Perspectives

Summary

Acute cardiovascular syndromes are a major cause of death in Western society and are generally triggered by rupture of an atherosclerotic plaque.[1, 2] Current treatment of atherosclerosis involves lipid lowering using statins, beta blockers, anti-thrombotic drugs, and life style advice. Even though improvements in treatment have led to a reduction in atherosclerosis-associated deaths and have led to an improved quality of life of patients, present treatment is inadequate to halt progression of cardiovascular disease with respect to plaque size (degree of occlusion) or to reverse existing plaques, and the number of people diagnosed with atherosclerosis still remains high. This indicates an urgent need for new therapeutic strategies to inhibit atherosclerosis and to prevent cardiovascular complications and acute syndromes.

Besides lipid accumulation, inflammation is considered a key process in atherosclerotic plaque development and in the pathogenesis of plaque rupture.[3, 4] Antigen presenting cells, such as dendritic cells and macrophages, play an important role in the inflammatory process within atherosclerotic plaques and are responsible for presentation of atherosclerosis-related antigens, such as oxLDL and HSP60, resulting in the attraction and activation of T cells.[5, 6] Upon activation T cells produce large amounts of pro-atherogenic cytokines that contribute to both the growth and destabilization of lesions, which can result in rupture of the lesion leading to thrombus formation and cardiovascular complications. T cells can be divided into several T cell subsets that can either be pro-inflammatory/pro-atherogenic, such as Th1 and Th2 cells, or anti-inflammatory/anti-atherogenic, such as regulatory T cells.

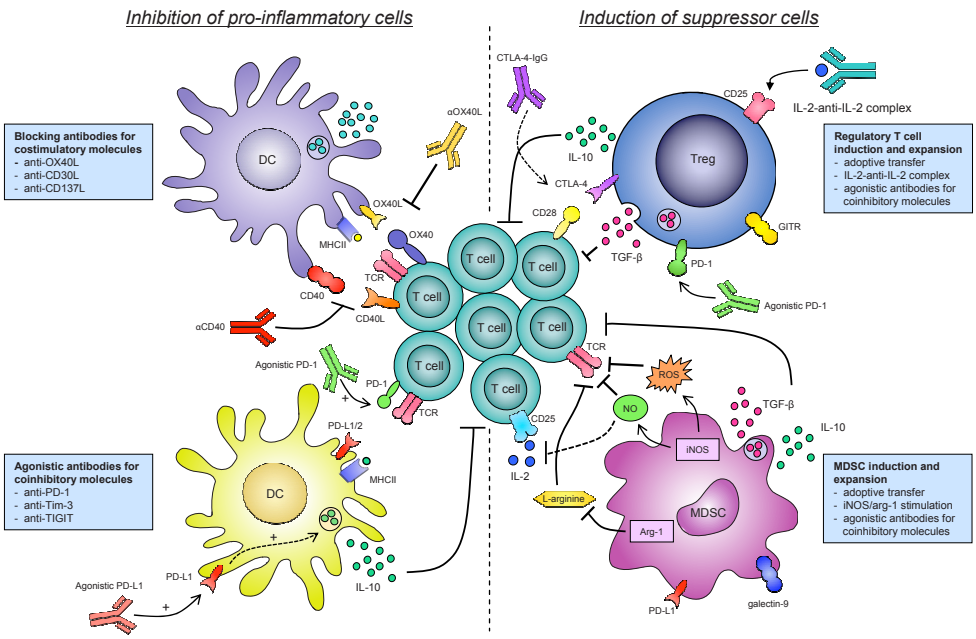

Figure 1. Pro-atherogenic T cells can be inhibited by blocking antibodies for costimulatory molecules or by agonistic antibodies for coinhibitory molecules and through the induction of suppressor cells, such as Tregs and MDSCs. Examples of possible treatments are provided in the blue boxes.

An imbalance between pro- and anti-inflammatory cells exists in atherosclerosis, with increased numbers of the first. Therefore, restoration of this balance by (1) inhibition of pro-inflammatory responses or by (2) inducing suppressor cells has great therapeutic potential to prevent cardiovascular disease (Figure 1). In this thesis, several therapeutic strategies to restore the balance of pro- and anti-inflammatory immune responses in atherosclerosis were investigated. In **Chapter 3-6**, modulation of costimulatory and coinhibitory pathways, a network of ligands present on antigen presenting cells that bind to their corresponding receptors on T cells and can either promote or inhibit immune cell function, was evaluated. In **Chapter 7-9**, the protective role of regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) was studied.

Modulation of costimulatory and coinhibitory pathways as a treatment of atherosclerosis

The immune system provides a large diversity of costimulatory and coinhibitory pathways and each pathway has its own unique effect on the fate of individual immune cells. Costimulatory signals can promote T cell survival, cell cycle progression and differentiation of naive T cells to effector and memory T cells, whereas coinhibitory molecules can terminate these processes directly or indirectly via the induction of Tregs. In this thesis we performed several studies that addressed the role of several costimulatory and coinhibitory pathways in atherosclerosis.

The costimulatory pathway formed by OX40 and OX40L is involved in the proliferation and survival of T cells, particularly Th2 cells, drives isotype switching of B cells, and is associated with cardiovascular disease incidence. In **Chapter 3**, we investigated the effect of OX40-OX40L interference on the regression of atherosclerosis in LDLr$^{-/-}$ mice by a combined anti-inflammatory (10 week treatment with anti-OX40L) and lipid-lowering strategy (switching to chow diet after 10 weeks of Western-type diet). Lipid lowering alone increased lesion stability without reducing lesion size, while additional anti-OX40L treatment also induced lesion regression. Treatment with anti-OX40L reduced circulating OX40-expressing CD4^{+} T cells and adventitial T cells.

In line with previous findings, interruption of the OX40-OX40L pathway reduced the Th2 response, as shown by decreases in GATA-3, IL-4 and IL-10 expression.[7] Notably, we demonstrate that the production of another typical Th2 cytokine, IL-5, was increased in B1 cells and T cells. B1 cells are dependent on IL-5 and enhance the secretion of atheroprotective natural oxLDL-specific IgM antibodies.[8] Anti-OX40L-treated mice had increased numbers of B1 cells and increased oxLDL-specific IgM titers. T cells that produce IL-5, independently of IL-4, can be induced by IL-33, which previously has been shown to be protective in atherosclerosis by inducing IL-5 and anti-oxLDL-specific IgM antibody formation[9], and by inhibiting foam cell formation.[10] We observed increased IL-33 expression in the spleen of anti-OX40L-treated mice and showed that IL-33 production is dose-dependently increased by anti-OX40L treatment of DCs and macrophages exposed to oxLDL. The increase in the atheroprotective factors IL-

33, IL-5 and oxLDL-specific IgM in anti-OX40L-treated mice likely contributed to the observed regression of atherosclerosis.

Another pathway through which OX40-OX40L blockade may facilitate lesion regression is via reduced IgE levels and subsequent reduced mast cell numbers and activation. IL-4 induces isotype switching of B cells from IgM- to IgE- and IgG-producing cells. Previously, we showed that anti-OX40L treatment reduced IgG1 levels[7] and we now show that interruption of OX40-OX40L treatment also induced a strong reduction in serum IgE. Activated mast cells are found in the adventitia of vulnerable and ruptured lesions of patients suffering from myocardial infarction [11, 12], and mast cell numbers correlate with the incidence of plaque rupture and erosion.[11] Our lab has previously shown that mast cells also play a crucial role in plaque progression and destabilization *in vivo*.[13] In addition, enhanced IgE levels were observed in patients with unstable angina pectoris and in dyslipidemia[14, 15] and recently, Wang et al. showed that IgE promotes atherosclerosis in ApoE$^{-/-}$ mice.[16] In line with these findings, we suggest that the reduced IgE levels due to the anti-OX40L treatment contribute to the observed lesion regression.

An overview of the mechanism of anti-OX40L-mediated regression of atherosclerosis is shown in Figure 2. Finally, it must be noted that interruption of the OX40-OX40L pathway did not induce full regression of lesions. Further research into modulating immune responses to induce regression must be explored and, in combination with lipid lowering, may hold the key to therapies for cardiovascular patients with well-established lesions.

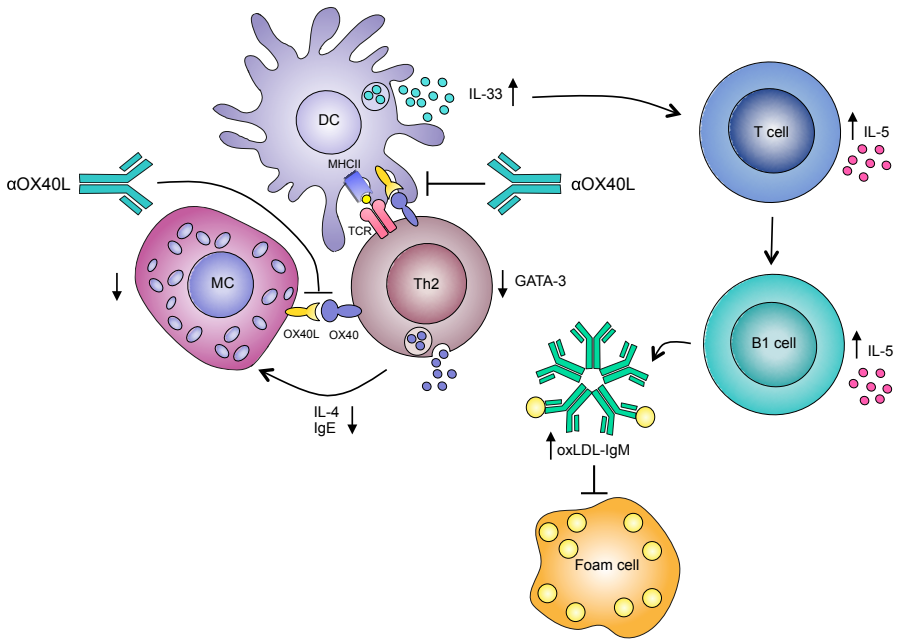

Figure 2. Schematic overview of the mechanism through which OX40-OX40L interruption can reduce atherosclerosis. Anti-OX40L treatment induces IL-33, IL-5 and oxLDL-specific IgM and reduces IL-4 and IgE levels, resulting in reduced mast cells numbers and activation.

Similar to OX40 and OX40L, CD30 and CD30L are members of the TNF(R) superfamily and are involved in the activation and proliferation of T and B cells. Whereas the CD30-CD30L pathway has been implicated in various autoimmune diseases, such as asthma[17], GVHD[18] and type I diabetes[19], no studies describe a role for the CD30-CD30L axis in atherosclerosis. In **Chapter 4** we therefore treated Western-type diet fed LDLr$^{-/-}$ mice with an anti-CD30L antibody for 8 weeks, which resulted in a reduction in atherosclerotic lesion formation in the aortic root by 35%. This reduction in atherosclerosis coincided with reduced adventitial T cell numbers, reduced percentages of CD4^{+} T cells in the spleen and lymph nodes and with strongly reduced splenocyte proliferation. In particular, CD4^{+} T cells isolated from anti-CD30L-treated mice proliferated less vigorously after αCD3/CD28 stimulation than CD4^{+} T cells from control mice, whereas their adhesion and migration capacity remained unaffected. Although signaling via CD30-CD30L may also affect humoral responses and mast cell activity, we did not detect a significant difference in immunoglobulin production and mast cell activity in anti-CD30L-treated mice compared with control mice. We conclude that the CD30-CD30L pathway solely exerts its function via inhibition of T cell responses in atherosclerosis, which identifies anti-CD30L treatment as a novel therapeutic modality in the inhibition of atherosclerotic lesion development and the prevention of acute cardiovascular syndromes.

In **Chapter 5**, we studied the role of T cell immunoglobulin and mucin domain 3 in atherosclerosis. Tim-3 is a coinhibitory type I transmembrane protein, which affects the function of several immune cells involved in atherosclerosis, such as monocytes, macrophages, effector T cells and Tregs. It has been reported that patients with atherosclerosis have increased Tim-3^{+} NK cells compared with healthy controls.[20] In line with these findings, we observed that Western-type diet feeding increased the percentage of Tim-3^{+} NK cells in blood of LDLr$^{-/-}$ mice, but also increased the percentage of Tim-3^{+} monocytes and DCs, cell types that largely contribute to the inflammatory process of atherosclerosis. When we treated Western-type diet fed LDLr$^{-/-}$ mice with an anti-Tim-3 antibody for 8 weeks, atherosclerotic plaque formation was increased with 35% in the aortic root and with 50% in the aortic arch. Although lesion stability did not differ between anti-Tim-3-treated mice and control mice, lesions of anti-Tim-3-treated mice contained significantly more macrophages than lesions of control mice. This might be the consequence of an increased influx of monocytes into the arterial wall, since we observed increased circulating monocytes in anti-Tim-3-treated mice. In addition, oxLDL-loaded macrophages treated with anti-Tim-3 secreted higher levels of MCP-1 *in vitro*, which might suggest that MCP-1 secreted by foam cells in the atherosclerotic lesions of anti-Tim-3-treated mice attracts monocytes to the inflamed arterial wall. These data are in line with studies by Monney and Frisancho-Kiss et al. who show that anti-Tim-3 treatment increases macrophage numbers and activation in mouse models of EAE[21] and inflammatory heart disease.[22] Additionally, we showed that anti-Tim-3 administration increased CD4^{+} T cells and reduced percentages of IL-10 producing Tregs and Bregs. Tim-3 has previously been associated with Tregs, as

blocking Tim-3 enhances type 1 diabetes in NOD-mice and prevents the generation of immunological tolerance in a transplantation model by dampening the function of Tregs.[23] To conclude, inducing Tim-3 signaling could provide a novel approach to inhibit pro-atherogenic immune responses.

To evaluate the contribution of another coinhibitory molecule, T cell immunoreceptor with Ig and ITIM domains (TIGIT), to atherosclerosis, we used an agonistic anti-TIGIT antibody as described in **Chapter 6**. Signaling via TIGIT directly inhibits T cell activation and proliferation through downregulation of the T cell receptor[24, 25] but can also induce IL-10 producing tolerogenic DCs upon binding to the poliovirus receptor (PVR).[25, 26] Several studies have shown that TIGIT is essential for T cell function in mice and humans and is mainly expressed on activated $CD4^+$ T cells.[25, 27] In line with these findings, we observed that TIGIT was upregulated on $CD4^+$ T cells from Western-type diet fed $LDLr^{-/-}$ mice in comparison with chow diet fed $LDLr^{-/-}$ mice and was further enhanced after αCD3/CD28 stimulation. Furthermore, we showed that agonistic anti-TIGIT greatly inhibited T cell activation and proliferation. This TIGIT-mediated downregulation of T cell responses inhibited several diseases such as EAE, collagen-induced arthritis and GVHD.[24, 25] Surprisingly, we observed that treatment of $LDLr^{-/-}$ mice fed a Western-type diet for 4 or 8 weeks with agonistic anti-TIGIT did not reduce atherosclerosis development and did not affect lesion composition. Possibly, the reduced T cell function was counteracted by enhanced activity of dendritic cells, which were elevated and expressed higher levels of MHC II and CD40 in agonistic anti-TIGIT-treated mice. Since TIGIT normally binds to PVR expressed on DCs to induce a tolerogenic phenotype, and agonistic anti-TIGIT blocks TIGIT-PVR signaling, this may explain a more pro-inflammatory phenotype of DCs in agonistic anti-TIGIT-treated mice. Despite agonistic anti-TIGIT treatment did not affect atherosclerosis, the TIGIT-PVR pathway could still be of interest to modulate pro-inflammatory immune responses in atherosclerosis and other autoimmune diseases.

Cellular targets of immune regulation to treat atherosclerosis

Another approach to regulate pathogenic immune responses in atherosclerosis is to promote suppressor cells, such as Tregs and myeloid-derived suppressor cells (MDSCs). Tregs play an important role in the regulation of T cell-mediated immune responses through suppression of T cell proliferation and cytokine production. Impaired Treg function has been associated with the pathogenesis of numerous diseases and in atherosclerosis, an imbalance between pro-inflammatory/pro-atherogenic cells (Th1/Th2) and Tregs exists.[28, 29] Therefore, increased Treg numbers may be beneficial for patients suffering from atherosclerosis. The role and therapeutic potential of Tregs in atherosclerosis has been the subject of intense investigation. Adoptive transfer of $CD4^+CD25^+$ T cells causes a reduction in atherosclerotic lesion development[30] while a depletion of $CD4^+CD25^+$ T cells aggravates lesion development.[30] Furthermore, van Puijvelde et al. showed that induction of antigen-specific Tregs via oral tolerance induction against oxLDL and HSP60 inhibits the initiation and progression of

atherosclerosis.[31, 32]

Tregs originate from the thymus and are characterized by the expression of the surface molecules CD4 and CD25, and expression of the transcription factor Forkhead box protein 3 (Foxp3).[33] Tregs may also acquire Foxp3 after oral tolerance induction. To study the effect of the elimination of $Foxp3^+$ Tregs in atherosclerosis, we used a novel vaccination strategy directed against Foxp3 expressing cells. The effect of Foxp3 specific elimination on initial and advanced atherosclerosis is described in **Chapter 7**. A dendritic cell based vaccine was used to provoke a cytotoxic T cell response against Foxp3 expressing cells. During the time course of the experiment we observed significant less $Foxp3^+$ Tregs in the circulation and lymphoid tissues of Foxp3 vaccinated mice. Furthermore, vaccination against Foxp3 aggravated atherosclerotic plaque formation in both initial and advanced atherosclerosis. This increase in lesion size was associated with increased cellularity, which may result from impaired inhibition of pathogenic T cells within the plaque upon depletion of Treg cells. Additionally, splenocytes from Foxp3 vaccinated mice proliferated more vigorously than splenocytes from control mice, which is indicative for a reduced number of Tregs since these cells suppress effector T cells. The results from this study further established the role of $Foxp3^+$ Tregs in atherosclerosis and are in line with the results of Ait-Oufella et al.[30]

Tregs have been shown to depend on IL-2 for optimal growth and survival.[34-37] Recently, it is shown that repeated injections of an IL-2 complex consisting of recombinant IL-2 and a specific anti-IL-2 monoclonal antibody expands Tregs[38], which very potently induces resistance to EAE and suppressed graft rejections[39], type I diabetes[40], murine-asthma[41] and myasthenia gravis.[42] The protective role of Tregs in atherosclerosis has been extensively investigated, however, only a modest increase in Treg numbers was achieved in the order of 1.5- to 2-fold, mostly for 2-3 weeks. In **Chapter 8** we therefore determined the effect of a vast IL-2 complex-mediated expansion of Tregs on the initiation and regression of well-established lesions. Administration of the IL-2 complex resulted in a 10-fold increase in Tregs in blood of $LDLr^{-/-}$ mice, which potently suppressed effector T cells and reduced initial atherosclerosis with 39%. Furthermore, we observed an increase in gene expression of IL-10 in the spleen and increased IL-10 secretion by Tregs, which suggests that IL-2 complex expanded Tregs exert their suppressive capacity via IL-10. These data are in line with previous reports in which IL-2 complex expanded Tregs suppressed airway inflammation[41] and EAE in an IL-10 dependent manner.[39] The increase in IL-10 in splenocytes of IL-2 complex treated mice may at least partially be responsible for the decrease in lesion size since IL-10 is protective in atherosclerosis.[43-45]

We also determined the role of IL-2 complex expanded Tregs in a more clinically relevant regression model of atherosclerosis, as most of the cardiovascular patients already have well-established lesions. Despite high Treg levels, no lesion regression was found in the IL-2 complex-treated group. However, we observed that Treg induction increased lesion stability as indicated by increased collagen content in the lesions.

Additionally, a 60% increase of adventitial $CD3^+$ T cells within lesions of IL-2 complex treated mice was observed, which most likely are the IL-2 complex-expanded Tregs. These data suggest a differential role for Tregs in different stages of atherosclerosis. Where Tregs inhibit lesion development during initial stages of atherosclerosis, they are important in the stabilization of well-established lesions during regression.

Besides Tregs, MDSCs also strongly suppress T cell responses. MDSCs are a population of early myeloid progenitor cells and immature myeloid cells that expand in the bone marrow under various pathological conditions and accumulate in lymphoid and non-lymphoid tissues where they serve a negative feedback function.[46] In contrast to cancer where expanded MDSCs aggravate the disease by the suppression of cytotoxic T cells, MDSC activity is highly appreciated in autoimmune diseases where the unwanted activation of the immune system needs to be suppressed. Although accumulating evidence implicates MDSCs as potent suppressors of several autoimmune diseases[47-51], the role of MDSCs in atherosclerosis is unknown.

In **Chapter 9** we isolated bone marrow-derived $CD11b^+Gr\text{-}1^+$ cells (MDSCs) from $LDLr^{-/-}$ mice in which the immune system was boosted by feeding a high-fat diet for 2 weeks.[28] These high-fat diet-associated MDSCs potently suppressed *in vitro* T cell proliferation in an IFN-γ/iNOS-dependent manner without inducing T cell apoptosis. Adoptive transfer of MDSCs into $LDLr^{-/-}$ mice fed a high-fat diet for 6 weeks ameliorated atherosclerosis with 35%. No differences were observed in collagen and macrophage content of the lesions. This reduction in atherosclerosis formation was associated with increased percentages of naive T cells and decreased percentages of effector T cells in the spleens of mice that received MDSCs. More specifically, MDSC-treated mice showed a 50% reduction in splenic Th1 and Th17 cells, subsets that are generally considered pro-inflammatory in atherosclerosis.[52-54] Additionally, adoptive transfer of MDSCs reduced B cell percentages, in particular pro-atherogenic B2 cells, possibly as a consequence of impaired proliferative capacity.

In conclusion, our study describes a novel cellular therapy using MDSCs to inhibit atherosclerosis. However, MDSCs can be subdivided in monocytic-MDSCs, induced by Th1 signals and expressing iNOS, or granulocytic-MDSCs, induced by Th2 signals and expressing arg-1, and whereas we adoptively transferred both monocytic and granulocytic MDSC subsets, it seems that monocytic-MDSCs are most likely responsible for the observed inhibition of atherosclerosis. In the Perspectives section, future approaches are described to investigate the relative potency of monocytic and granulocytic MDSCs to provoke immune suppression in atherosclerosis.

Perspectives

In this thesis, several mechanisms to regulate pathogenic immune responses in atherosclerosis have been studied. T cells play a major role in the pathogenesis of atherosclerosis by promoting inflammation and destabilizing advanced lesions. T cells are regulated by a network of costimulatory and coinhibitory molecules and by several suppressor cells such as Tregs and MDSCs. These pathways of immune regulation form potent candidates for an immunotherapy of atherosclerosis. Novel therapeutic strategies to treat atherosclerosis are needed since death from cardiovascular diseases continues to increase worldwide, despite the use of statins, anti-thrombotic drugs, and anti-hypertensive treatment.[55]

Modulation of costimulatory and coinhibitory pathways

In cardiovascular disease, modulation of costimulatory and coinhibitory molecules can be a powerful tool to target specific stages of atherosclerosis or specific cell types involved in the pathogenesis of atherosclerosis. A highly relevant feature of costimulatory and coinhibitory pathways is that they individually have a unique effect on the behaviour of specific immune cells and thus on the outcome of disease. As shown in **Chapter 3** and **4**, interference of OX40-OX40L and CD30-CD30L signaling both reduce atherosclerosis via different pathways; anti-OX40L specifically targets Th2 responses and mast cell activity, whereas anti-CD30L limits all $CD4^+$ T cell responses without affecting a specific T cell subset or other immune cells. In addition, several costimulatory and coinhibitory molecules are involved in the induction and function of Tregs. This enables the development of a treatment that particularly targets different subsets of T cells. Ultimately, it would be ideal to modulate antigen-specific pro-atherogenic T cells with blocking costimulatory antibodies and agonistic coinhibitory antibodies without affecting all T cells and other immune cells to limit any adverse effects on the immune system. Although several candidates of atherosclerosis specific antigens have been investigated, such as oxLDL, HSP60 and ApoB100, to date the exclusively atherosclerosis-associated antigen is not identified yet, which makes it difficult to specifically target the pro-atherogenic T cells. However, some costimulatory molecules, such as OX40, are virtually absent on naive T cells but are upregulated on activated T cells. Targeting these costimulatory molecules with blocking antibodies could specifically eliminate the pathogenic T cells without causing any side effects. Furthermore, the ligand of OX40, OX40L, is expressed on endothelial cells, which upon blockade can also reduce the attraction of $OX40^+$ T cells to the site of inflammation.

Blocking and agonistic antibodies for costimulatory and coinhibitory molecules have already been extensively explored in cancer and allograft rejections. Blocking antibodies for CTLA-4 and PD-1 to boost T cell responses are approved for treatment of patients with several types of cancer.[56] In contrast to cancer where T cell activity is highly appreciated, the unwanted activation of the immune system needs to be suppressed in atherosclerosis. Therefore, whereas in cancer for example a blocking

PD-1 antibody to promote T cell activity is beneficial, in atherosclerosis an agonistic PD-1 antibody is needed to suppress T cells. CTLA-4-Ig has already been established as an effective treatment for human autoimmune diseases including rheumatoid arthritis[57] and psoriasis.[58] At present, one clinical trial has been completed using anti-OX40L in the prevention of allergen-induced airway obstruction in adults with mild asthma.[59] However, no study results are reported yet.

Although many antibodies against costimulatory and coinhibitory molecules have been approved and are used in clinical settings, caution is needed when translating animal experiments to the clinic, as a Phase I clinical trial with an agonistic monoclonal anti-CD28 antibody induced a strong cytokine storm (IFN-γ, TNFα, IL-2) several hours after drug infusion, which caused multiorgan failure in six human volunteers who ended up on the intensive care unit.[60] Moreover, blocking costimulatory pathways and stimulating coinhibitory pathways may enable opportunistic infections to emerge. However, treatment can be adjusted in a way that patients will only receive blocking antibodies for costimulatory molecules or agonistic antibodies for coinhibitory molecules temporarily until the lesion is stabilized.

No clinical trials investigating antibodies for costimulatory and coinhibitory molecules in cardiovascular disease have been started yet. In fact, only recently the first clinical trial involving interference of inflammatory pathways to reduce major cardiovascular events in persons with pre-existing coronary artery disease was launched.[61, 62] This CANTOS trial is a large-scaled study in which over 17.000 subjects will be included to test three different doses of Canakinumab, a humanized monoclonal antibody specific for IL-1β, compared with placebo. Canakinumab is already approved in other autoimmune diseases where IL-1β plays a major role, such as Muckle-Wells syndrome and familial cold autoinflammatory syndrome.[63] This study will provide the first evidence whether interference in specific inflammatory pathways can reduce clinical events in cardiovascular patients and will possibly initiate numerous clinical trials focused on modulating immune responses in atherosclerosis.

Interestingly, some anti-tumor therapy studies have indicated that treatment with only a single costimulatory agonist, in addition to existing cytostatic therapy or cancer-antigen vaccination, is not effective or induces adverse immunological events. The co-administration of a second agonist or another factor that stimulates T cell function is necessary to achieve a greater anti-tumor reactivity. For example, a clinical trial with an anti-CTLA-4 antibody (MDX-010) in conjunction with anti-cancer antigen vaccination resulted in regression of cancer but unfortunately also induced severe autoimmune diseases in melanoma patients.[64] Kocak et al. showed that a combination therapy in mice with pre-existing tumors with anti-CTLA-4 and anti-4-1BB enhances anti-tumor immunity without any adverse effects on the immune system.[65] The mechanism through which the combination of anti-CTLA-4 and anti-41BB reduce each other's side effects is not fully explained but it is shown that they synergistically enhance the suppressive capacity of regulatory T cells. Currently, a phase I clinical

trial is carried out in which anti-CTLA-4 (Ipilimumab) is combined with anti-PD-1 (BMS-936558) to treat melanoma patients.[66]
It may be very likely that a combinatorial therapy may also be very effective in atherosclerosis. For example, previous studies reported a synergistic effect of OX40L and CD30L on T cell responses. Blocking CD30 together with OX40 signaling prevented lethal X-linked CD4 T cell-dependent Th1- and Th2-driven autoimmune disease in mice lacking regulatory T cells[67] and affected effector and memory T cell formation and function.[68] Moreover, a combined blockade of costimulatory signals, e.g. anti-OX40L or anti-CD30L, with activation of coinhibitory signals, e.g. PD-1 or Tim-3 agonists, could be explored to suppress for example pro-atherogenic T cells while stimulating athero-protective Tregs. More research should be performed to identify the most relevant combinations of blocking and agonistic antibodies for costimulatory and coinhibitory molecules respectively, which could be used as an immunotherapy to inhibit atherosclerosis.

Costimulatory and coinhibitory molecules are also essential for the establishment and maintenance of immunological tolerance via the induction of tolerogenic DCs and Tregs. A frequently used method to induce tolerance is by oral immunization. In atherosclerosis, oral tolerance induction to oxLDL[31], HSP60[32], β2-glycoprotein I[69] and ApoB100 peptide[70] has been shown to suppress atherosclerosis. Van Puijvelde et al. showed that oral tolerance induction against oxLDL and HSP60 increased Tregs and their CTLA-4 expression.[31, 32] Possibly, oral tolerance induction against auto-antigens, such as oxLDL, can be combined with blocking antibodies against costimulatory molecules or agonists for coinhibitory molecules to achieve T cell non-responsiveness against these auto-antigens and to promote the induction of antigen-specific Tregs.

Treg-based cell therapy

As shown in **Chapter 7** and **8**, Tregs are also efficient regulators of pathogenic immune responses and the usage of Tregs as a therapeutic agent shows great potential in the treatment of atherosclerosis. In several diseases the number or function of Tregs is decreased and restoring the balance between Tregs and pro-inflammatory cells may be beneficial. Therefore, a lot of research is nowadays focussed on the development of $Foxp3^+$ regulatory T cells (Figure 3). One possible treatment strategy is an adoptive transfer of Tregs. This procedure will require an enormous quantity of Tregs and can be achieved by isolation of $Foxp3^+$ T cells from the blood of a patient and subsequent *ex vivo* expansion to obtain large numbers for therapy.[71, 72] Two Phase I clinical trials have tested the ability of *ex vivo* expanded Tregs to prevent GVHD after allogeneic bone marrow transplantation.[73, 74] This Treg cell-based therapy proved to be safe and reduced GVHD. Moreover, new clinical trials are starting in which the safety and effectiveness of Treg cell-based therapy is tested in individuals with autoimmune diseases such as type 1 diabetes[75] and in organ transplantation patients.[76]
Alternatively, Tregs can be expanded *in vivo*. This can be achieved by targeting some

costimulatory and coinhibitory molecules as described previously but also with an IL-2 complex consisting of recombinant IL-2 and anti-IL-2. Besides effectively reducing atherosclerosis (**Chapter 8**), this IL-2 complex very potently induced resistance to EAE and suppressed graft rejections[39], type I diabetes[40], murine-asthma[41] and myasthenia gravis[42] in mouse studies. Future research should reveal whether administration of this IL-2 complex would also be beneficial in patients with cardiovascular disease.

Although current experimental treatments and clinical trials are based on the expansion of aspecific Tregs, it may be of great interest to induce antigen-specific Tregs. Previously, oral tolerance induction against oxLDL and HSP60 inhibited atherosclerosis development via the induction of antigen-specific Tregs[31, 32] Possibly, oral tolerance induction can be combined with an IL-2 complex treatment to first induce antigen-specific Tregs and thereafter expand these Tregs.

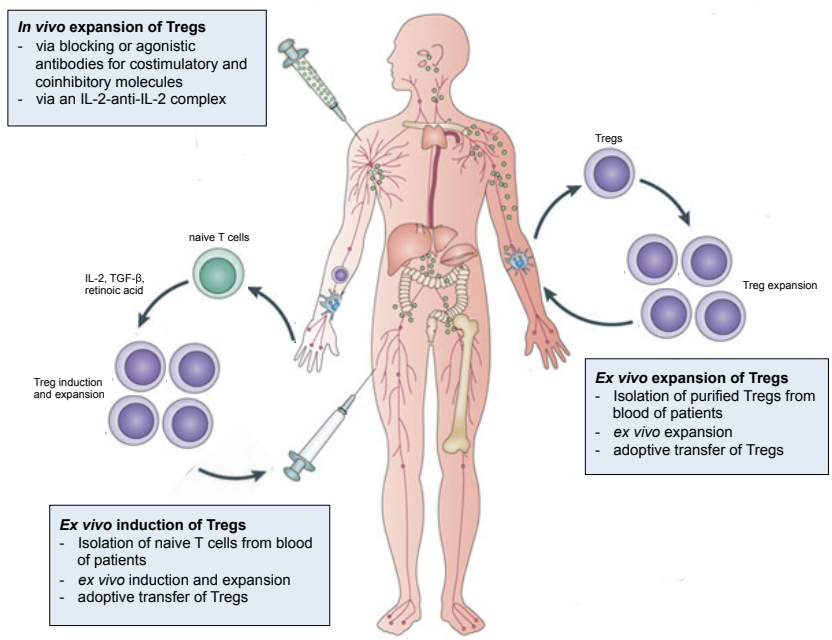

Figure 3. Schematic overview of different approaches to target Tregs for immunotherapy.

MDSC-based cell therapy

In atherosclerosis, elevated numbers of $CD11b^{+}Ly6G^{-}Ly6C^{hi}$ cells (inflammatory monocytes) and $CD11b^{+}Ly6G^{+}Ly6C^{low}$ cells (neutrophils) are considered pro-inflammatory and correlate to lesion size.[36, 37] However, in the cancer field cells with the exact same phenotype exert an anti-inflammatory function and are called monocytic-MDSCs and granulocytic-MDSCs, respectively. MDSCs are known to expand in the bone marrow of diseased individuals and migrate into several lymphoid and non-lymphoid tissues. Elevated levels of circulating MDSCs are found in patients with cancer, multiple sclerosis and rheumatoid arthritis.[77-79] Further research is needed to

investigate whether MDSCs also accumulate in the blood of patients suffering from cardiovascular disease and to what extent these cells overlap with the inflammatory monocytes and neutrophils.

In **Chapter 9** we show that adoptive transfer of MDSCs (mo-MDSCs and gr-MDSCs) regulated T and B cell responses in an experimental model of atherosclerosis and inhibited lesion development. This shows the therapeutic potential of MDSCs as a novel immune-therapy to treat cardiovascular disease and opens an exciting new area of investigation. To fully characterize and comprehend the role of MDSCs in atherosclerosis multiple experiments should be performed. To determine the underlying mechanism of MDSC-mediated suppression, MDSCs can be isolated from Arg-$1^{-/-}$ and $iNOS^{-/-}$ mice, which lack functional gr-MDSCs and mo-MDSCs, respectively. To further determine the relative potency of mo-MDSCs and gr-MDSCs to inhibit atherosclerosis, each subset can be sorted with FACS or with magnetic bead labeling and subsequently adoptively transferred into Western-type diet fed $LDLr^{-/-}$ mice. Furthermore, since MDSCs respond to their microenvironment it is possible that they exhibit distinct biological activities depending on the microenvironment in the different pathological stages of atherosclerosis. It has also been shown that MDSC subpopulations from blood and tumors can differ in their capacity to mediate T cell suppression.[80] This raises the question whether MDSCs are present in lesions and whether MDSCs in lesions will have a different phenotype and suppression capacity in comparison with MDSCs present in the bone marrow and possibly in other sites.

Collectively, before MDSCs are considered as an innovative immunotherapeutic strategy to prevent atherosclerosis in cardiovascular patients, more research is required.

In conclusion, the research described in this thesis provided novel approaches to dampen the immune response in atherosclerosis. However, further characterization of these potential new drug targets and cellular therapies are necessary before they can be applied in clinical research.

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