General Discussion

Background

Cardiovascular diseases form the most common cause of death in the western world with atherosclerosis as the most common etiology *(1)*. Atherosclerosis is characterized by lipid deposition in the intima of medium to large-sized arteries, evoking pathogenic immune infiltration in the vessel wall causing low grade inflammation of the vessel wall *(2–4)*. Development of atherosclerotic lesions spans decades and can lead to the formation of large plaques which occlude the coronary vessel lumen to such an extent that during e.g. exercise, myocardial tissue is deprived of sufficient oxygen supply leading to symptoms such as shortness of breath, nausea and fatigue, and pressure and tightness of the chest *(5, 6)*. Although this situation of stable angina is just weakly correlated with cardiovascular death, it is associated with heart failure, increased hospitalizations, and reduced quality of life *(5)*. Besides stable angina, atherosclerosis can also lead to the formation of vulnerable lesions, which due to plaque rupture or plaque erosion induce sudden formation of a thrombus *(7, 8)*. The formed thrombus can restrict blood flow in situ, or can break away from the site of thrombus formation and clog an artery in the narrowing arterial tree *(7, 8)*. This can lead to acute oxygen deprivation and injury of downstream tissues and underlies the life-threatening conditions of myocardial infarction and ischemic stroke *(7, 8)*. Treatment of an acute thrombotic occlusion is aimed at restoring blood flow as quickly as possible to prevent ischemia mediated tissue damage *(7)*, by fibrinolytic treatment or surgical removal of the clot *(9)*. Vulnerable or occlusive atherosclerotic plaques can be surgically removed through endarterectomy *(10)*. Impaired blood flow due to narrowed artery lumen can be restored through angioplasty and stent placement opening up the vessel lumen, or by bypass surgery redirecting blood flow *(10)*. Restenosis is a frequently occurring phenomenon after cardiovascular surgery, making repeated interventions sometimes necessary *(11)*.

Current treatment regimens to prevent progression of atherosclerosis and (re)occurrence of major cardiovascular events are predominantly aimed at normalizing lipid levels through adopting a healthy lifestyle, and pharmacologically by use of lipid lowering drugs such as statins *(12)* and PCSK9 inhibitors *(13)*. Lowering lipid levels reduces the risk of a cardiovascular event, however in many patients statins are not well tolerated, or despite successful reduction in lipid levels, still a residual risk for a cardiovascular event is present due to unresolved inflammation *(14)*. The recent success of the CANTOS trial, reducing major cardiovascular events through administration of a monoclonal antibody neutralizing the pro-inflammatory cytokine IL-1β (Canakinumab) *(14–16)*, although not approved for the treatment of atherosclerosis by the FDA, implies that modulation of the immune system is also a feasible way of treating atherosclerosis and reducing cardiovascular risk in human. Following the paradigm that lowering inflammation can reduce atherosclerosis and prevent major cardiovascular events, clinical trials with low doses of the immunosuppressant methotrexate *(17)* and colchicine *(18)* were commenced. The results from treatment with low doses of the methotrexate were rather disappointing, as cardiovascular deaths were not

inhibited by methotrexate while negative side effects, including increased liver enzymes in circulation, a higher incidence of non-basal-cell skin cancers, and more prevalent mouth sores and oral pain, were also found in the methotrexate treated group *(19)*. Similarly, side effects for treatment with Canakinumab and colchicine are commonly reported. In experimental models of atherosclerosis, modulation of the antigen specific immune response towards plaque constituents, including albumin *(20)*, oxidized LDL *(21, 22)*, Apolipoprotein-B100 (ApoB100) *(23–28)*, collagen type VI *(29)*, and heat shock proteins *(30)*, have been capable of reducing atherosclerosis. These studies indicate that vaccination-based approaches against plaque antigens could be interesting to further explore for use in humans, and would likely result in fewer side effects than general immunosuppressants.

In this thesis

In this thesis we aimed to beneficially modulate the immune response to treat atherosclerosis. Hyperlipidemia and inflammation are driving factors behind atherosclerosis, the interactions between lipids and immune system are therefore reviewed in **Chapter 2**. A pivotal step in atherogenesis is the attraction and activation of macrophages in the subendothelial space due to lipid retention. The Oxidized and aggregated lipoproteins induce TLR4 mediated activation of macrophages, and in combination with signals derived from antigen specific Th1 cells promote macrophages to adapt a pro-inflammatory M1 phenotype *(31, 32)*. Due to secretion of pro-inflammatory cytokines and chemokines, and high MHC-II and co-stimulatory molecule expression, M1 macrophages attract other immune cells to the atherosclerotic lesion, sustain inflammation, and are capable of interacting with Th1 CD4 T cells and hereby have a detrimental effect in the context of atherosclerosis *(33, 34)*. During atherosclerosis the natural immune tolerance present to self-derived plaque antigens is broken, as was elegantly shown for collagen type V *(35)*. The ensuing autoimmune response against plaque antigens is considered to be mainly pro-atherogenic *(2, 3)* and is skewed towards a Th1 response *(3, 4)*, resulting in high IFN-γ and TNF-α levels in the atherosclerotic lesion *(36)*. Since immune responses against (ox)LDL in the context of atherosclerosis are well documented *(37, 38)*, we aimed to modulate the immune response against LDL in **Chapter 3-5** to treat atherosclerosis. In **Chapter 6** we aimed to inhibit the pathogenic Th1 response as a whole through immunoproteasomal inhibition, which surprisingly also affected lipid homeostasis and improved parameters of metabolic syndrome.

As atherosclerosis is marked by accumulation of (ox)LDL in the vessel wall leading to induction of auto-reactive (ox)LDL specific CD4 T cells *(37, 38)*, an attractive treatment option would be reinstating natural tolerance to LDL, reducing pathogenic inflammation. Regulatory CD4 T cells (Tregs) are pivotal for keeping immune tolerance and were found capable of keeping autoimmune Th1 immune reactions in check *(39, 40)*. Although Tregs can confer bystander immunosuppression, the immunosuppressive effect of antigen specific Tregs was found greater than that of polyclonal Tregs *(41)*. A means to induce antigen specific Tregs is

the administration of antigens through oral administration, which leads to antigen presentation by the intestinal tolerogenic $CD11C^{+}CD103^{+}$ DC population, which favors the induction of antigen specific immune suppressive regulatory B cells (Bregs), Th3, Tr1 and inducible Tregs (iTregs) *(42)*. Oral administration of oxLDL was previously found to reduce atherosclerosis through induction of oxLDL specific Tregs *(21)*, however induced Treg numbers quickly decline to baseline levels *(21, 43)*. To improve the therapeutic effect of oral oxLDL administration we hypothesized that we could first induce oxLDL specific Tregs through oral administration of oxLDL and then maintain the levels of oxLDL specific Tregs through expansion of the total Treg population. To specifically expand the Treg population IL-2 complexed to antibody clone JES6-1A12 was administered, allowing high affinity IL-2 receptor mediated growth and expansion of Tregs, but inhibiting binding to moderate and low affinity IL-2 receptors *(44)*. Also this clonal induction of Tregs was previously found to confer atheroprotection in our lab *(45)*.

Despite establishing elevated levels of Tregs after IL-2c treatment, coincident with lowered numbers of circulating immune cells indicative of immune suppression, only separate oxLDL treatment significantly reduced atherosclerosis. Separate IL-2c treatment and IL-2c treatment preceded by oxLDL treatment led to a trend towards atherosclerosis reduction compared to the control group, but appeared to be less effective than separate oxLDL treatment. Tregs are known to adapt to environmental cues to effectively inhibit different types of immune responses *(46–48)*. Moreover Tregs with distinct developmental origins, being thymus derived (nTregs) or the peripherally induced iTregs, have been described to have complementary and so, distinct functions. It is possible that differential spacial localization, or intrinsic differences between Treg populations cause differential expansion of Treg populations in response to IL-2c treatment. Moreover adaptive transfer of nTregs was found to reduce iTreg numbers *(49)*, implicating the existence of a feedback mechanism between the Treg populations, through which polyclonal expansion of non-oxLDL specific Tregs could have reduced oxLDL specific Tregs instead of expanding them. Lack of reliable markers for the different Treg populations *(50)* and difficulties in distinguishing antigen specific T cells make it very difficult to assess the effect of IL-2c treatment on the different Treg populations *in vivo*. It would be interesting to study the impact of the different Treg populations on atherosclerosis, and whether skewing of the Treg phenotype could impact atherogenesis.

The heterogeneous nature of LDL particles native LDL is not suitable for use in vaccinations. Therefore, several studies have been dedicated to finding immunogenic epitopes in ApoB100. A peptide library spanning the full ApoB100 protein was screened with human blood plasma for antibody binding to identify antibody epitopes in ApoB100 *(51)*. One of the peptides recognized by human serum derived antibodies is p210 (KTTKQSFDLSVKAQYKKNKH, 3163-3182), named after the peptide number in the peptide library *(51)*. Several vaccination strategies centered around p210 have been employed, successfully reducing atherosclerosis,

however the proposed mechanisms of action of p210 are divergent. P210 induced atheroprotection has been dedicated to induction of p210 antibodies *(27)*, Bregs *(52)*, CD4 Tregs *(25, 52)*, and CD8 T cells *(24, 53)*. To be therapeutically applicable, and to optimize the vaccine formulation and administration, it is important to identify the mechanism of action of vaccination with p210. Because p210 is a human ApoB100 derived sequence with 90% homology with the corresponding murine sequence we used a preclinical model with endogenous expression of human ApoB100, to allow normal thymic selection of T cell clones specific for human apoB100 and p210, and in vivo presentation of the cognate antigen for p210 specific T cell clones. Furthermore in all but one study *(54)* regarding p210 immunization in atherosclerosis, ApoE deficient mice were utilized. Since p210 is part of the LDL receptor binding site A in ApoB100 *(55, 56)*, we inquired whether the protective effect of p210 vaccination still was observed when no LDLr was present. Therefore we used LDLr deficient and human $ApoB^{100/100}$ transgenic (HuBL) mice *(56, 57)* in the experiments described in this paper.

As shown previously *(21)* and confirmed in **chapter 3**, induction of tolerance towards oxLDL reduces atherosclerosis. Therefore, we first aimed to induce a tolerogenic response against p210 through oral administration *(42)* of p210 coupled to cholera toxin B (CTB), known to promote mucosal uptake and tolerance *(58)*. As intranasal vaccination with CTB-p210 was previously reported to mediate atheroprotection through induction of regulatory B cells (Bregs) *(52)* and regulatory T cells *(25)*, we assessed the induction of Tregs and Bregs by flow cytometry. We indeed observed an increase in IL-10 producing Bregs in PMA and Ionomycins stimulated splenocyte cultures of CTB-p210 treated HuBL mice, but did not find increased Treg levels in various immune organs. The absence of Treg induction by oral CTB-p210 administration could be caused by the absence of a CD4 T cell epitope in p210, supported by lack of p210 binding to MHC-II ($I\text{-}A^b$) *(59)* and in line with in silico models of MHC-II binding *(59)*. CTB-p210 treatment did increase p210 IgG, which also had been described in previous studies *(25, 52)*. Despite induction of Bregs and p210 antibodies we did not observe a reduction in aortic root and brachiocephalic artery lesion size. In the only other published study in HuBL mice studying p210, intranasal CTB-p210 treatment was reported to lead to a trend towards reduced plaque formation measured by en face ORO staining of the aorta ($p = 0.059$) *(54)*. However, since the median lesion area of the control group was below 1% it is difficult to interpret these results.

Since the only effect of oral CTB-p210 administration we observed was induction of p210 IgG, we wanted to ensure that the absence of an effect on atherosclerosis by CTB-p210 vaccination was not due to an insufficient induction of p210 IgG levels. Therefore we performed another atherosclerosis study in which we first induced high levels of p210 IgG through vaccination with p210 coupled to pan DR epitope (PADRE) and adjuvanted with alum, and then switched the HuBL mice to a western type diet (WTD). With ELISA for p210,

we confirmed that p210 antibody levels remained high until sacrifice. As alum adjuvanted vaccination with p210, with cBSA as carrier protein, was used in a similar scheme reducing atherosclerosis in ApoE$^{-/-}$ mice *(24, 60, 61)*, reportedly via CD4 and CD8 T cells, we also assessed the induction of CD4 T cell and CD8 T cell populations upon PADRE-p210 vaccination. In accordance with binding predictions for p210 binding to murine MHC-I (H-2Kb and H-2Db) and MHC-II *(59)*, we did not observe T cell activation or induction of regulatory T cell subsets. Although we established high p210 antibody levels over the entire course of WTD feeding, we did not observe an effect on aortic root atherosclerotic lesion size or composition of PADRE-p210 immunization.

The lack of p210 antibody induced atheroprotection in our studies could be linked to the biological function of p210 in ApoB100, as it is part of the LDLr binding site A in ApoB100 *(55, 56)*. In line with p210 antibodies meddling with binding of ApoB100 to the LDLr, LDL uptake by cultured adipoctyes was inhibited by p210 antibodies *(62)*. In macrophage cultures incubated with oxLDL, addition of p210 antibodies inhibited the formation of foam cells but did not limit uptake of oxLDL *(62)*. As upregulation of cholesterol efflux gene expression in macrophages was observed after incubation with p210 IgG in another study *(27)*, it is possible that p210 IgG can improve lipid handling of macrophages. In vivo, immunization with p210, and administration of antibodies against MDA-modified p210 reduced atherosclerosis in ApoE$^{-/-}$ mice *(27)*, indicating that at least part of the atheroprotective effect of p210 vaccination in ApoE$^{-/-}$ mice is antibody derived. Also in humans, p210 IgM and IgG levels are correlated with improved carotid intima-media thickness parameters *(63–65)*, indicating atheroprotective properties of p210 antibodies in human. Interestingly the inverse correlations between anti-p210 IgG levels and baseline composite measures of carotid intima-media thickness disappeared when adjusted for known risk factors *(63)*, suggesting that improved lipid handling induced by anti-p210 antibodies *(27, 62)* might also occur in human.

Besides induction of protective antibodies, the protective effect of vaccination with p210 formulations has been dedicated to the induction of atheroprotective T cell populations *(24, 25, 53)*. As mentioned, we did not detect induction of CD4 T cells or CD8 T cells, in line with MHC binding and prediction. Neither did we detect an atheroprotective effect which, if T cell dependent, should not have been affected by the use of an LDLr$^{-/-}$ model or ApoE$^{-/-}$ model. This suggests that T cell responses induced by p210 vaccination are likely based on an indirect mechanism. Adjuvant properties of p210 could explain the divergent immunological effects which have been described upon administration of different p210 formulations. Actually, the heparan sulfate proteoglycan and LDLr binding properties of the LDLr binding sites of ApoB100 were used to enhance uptake of the SIINFEKL peptide, promoting cross priming of CD8 T cells *(55)*. Furthermore, p210 coupling to FITC enhanced FITC uptake by DCs *(24)*, showing that p210 can promote uptake of coupled proteins. In splenocyte cultures incubated with CTB-p210 but not with p210 or CTB, we observed enhanced cell death independent of the treatment group (control, CTB, CTB-p210). As flow cytometric analysis of CTB-p210

incubated cultures did not reveal activation of cytotoxic CD4 or CD8 T cells, this suggest that p210 reinforced the known pro-apoptotic properties of CTB *(66)*. Similarly, CTB-p210 was found to induce significantly higher levels of Bregs in culture than CTB-OVA *(52)*. Many of the studies centered around vaccination with p210 have used cBSA as a carrier protein without proving antigen specificity of the T cell responses to which the observed atheroprotection was dedicated. As immunization with cBSA itself using alum was found to reduce atherosclerosis *(20)*, it is possible that p210 promoted the atheroprotection observed in combined p210 and cBSA immunization. Vaccination with cBSA, and the 70% homology between bovine albumin and murine albumin (accession M73993.1 vs accession BC049971.1), could have led to cross-reactive antigen specific responses against murine albumin, which is present in large quantities in atherosclerotic plaques *(67)*. The possibility that treatment of atherosclerosis with ApoB100 derived CD4 T cell epitopes is feasible was shown by immunization (CFA used for priming, IFA for booster) with ApoB100 derived peptides, predicted to bind I-A^b and thereby capable of inducing CD4 T cells *(68)*. The enhanced IL-10 expression in aorta's of ApoB100 peptide vaccinated mice suggest that regulatory CD4 T cells were induced by vaccination although the overall FoxP3 cell levels were not increased *(68)*. It would be interesting to assess whether this tolerogenic response towards ApoB100 peptide vaccination could still observed when vaccination was commenced in later stages of atherosclerosis development when immune tolerance towards plaque antigens like ApoB100 might have been eroded, as was observed for collagen type V *(35)*. Induction of CD4 Treg mediated tolerance towards plaque antigens seems a promising strategy to specifically inhibit the atherosclerotic immune response. To be effective in advanced stages of atherosclerosis, probably mucosal administration of human MHC-II binding epitopes of plaque antigens in combination with tolerogenic adjuvants, like CTB, would be required.

Much less is known about the relevance of CD8 T cells in the context of atherosclerosis, although CD8 T cells are present in large quantities in the atherosclerotic plaque *(69)* and have an activated phenotype *(70)*. CD8 T cells are specialized at killing of specific target cells, mediated through T cell receptor (TCR) interaction with an MHC-I/peptide complex present on the target cell. Depending on target antigen, likely reflecting which cell types were targeted, induction of antigen specific CD8 T cell responses were found to be atheroprotective *(24, 71, 72)* or atherogenic *(73)*. As ApoB100 is considered one of the main plaque atherosclerotic plaque antigens, we aimed to assess the role of ApoB100 specific CD8 T cells in atherosclerosis. Cross-presentation of plaque constituents, including ApoB100, by APCs could make plaque APCs subject to killing by ApoB100 specific CD8 T cells. Because suppressed macrophage apoptosis results in increased atherosclerosis *(74, 75)*, we hypothesized that induction of ApoB100 specific CD8 T cells, presumably promoting killing of plaque macrophages by CD8 T cells, could be therapeutically relevant. Therefore, we opted to test this hypothesis with ApoB100 derived human MHC-I (HLA-A2) restricted epitopes to be directly applicable in human, in **chapter 5**. With *in silico* prediction tools of peptide

processing and HLA-A2 binding *(76–85)*, we selected 6 ApoB100 derived peptides to be synthesized. HLA-A2 binding was confirmed for all 6 epitopes in T2 cell binding assays, and immunogenicity was confirmed for 5 peptides by vaccination with peptide pulsed HLA-A2 transgenic DCs in HLA-A2 transgenic mice (HHD mice) *(86)*, deficient for murine MHC-I and human ApoB100. For the atherosclerosis studies HHD mice and HuBL mice were crossbred to generate HLA-A2 and human ApoB100 transgenic mice, deficient for the LDLr to allow atherosclerosis development, and either with normal expression (HuBL-A2^{m+}) or devoid of murine MHC-I expression (HuBL-A2^{m-}). CD8 T cells were again primed with a mixture of peptide pulsed HHD DCs, but then boosted after a week with peptide adjuvanted with poly(I:C) and αCD40. This vaccination regimen was previously reported to induce neo-epitopes specific CTLs that were effective in penetrating and killing tumors, indicating that this vaccination approach yields migratory and functional CTLs *(87, 88)*. For ApoB$_{406\text{-}414}$, ApoB$_{3070\text{-}3078}$, and ApoB$_{4531\text{-}4539}$, recall responses were detected in the spleens of ApoB100 peptide vaccinated HuBL-A2^{m-} and HuBL-A2^{m+} mice 8-9 weeks after booster vaccination at sacrifice, indicating successful vaccination. Interestingly no CD8 T cell recall response could be detected for ApoB$_{406\text{-}417}$ and ApoB$_{2356\text{-}2364}$ in the ApoB100 peptide treated HuBL-A2^{m-} and HuBL-A2^{m+} mice, suggesting thymic negative selection or peripheral tolerance induction towards ApoB$_{406\text{-}417}$ and ApoB$_{2356\text{-}2364}$. Recall responses towards pooled peptides were also assessed in cultures from mediastinal lymph nodes and aortic arches from HuBL-A2^{m-} mice. Also, in cultures of cells from mediastinal lymph nodes recall responses were observed, however not in aortic arch derived cells. As enhanced effector CD8 T cells in the blood and CD8 T cell levels in the aortic arch cultures were found due to ApoB100 peptide vaccination HuBL-A2^{m-} mice, it is very likely that ApoB100 peptide specific CD8 T cells homed to the atherosclerotic lesion. Since the ApoB100 peptides can be externally loaded on MHC-I, so without need for cross-presentation, the absence of recall responses in the aortic arch cultures could also not be dependent on defective cross-presentation. These data therefore suggest that CD8 T cell activation in to response to TCR stimuli is reduced in the plaque environment. Reduced responsiveness could be CD8 T cell intrinsic, e.g. due to chronic antigen exposure in the plaque leading to CD8 T cell exhaustion *(89, 90)*. In line with CD8 T cell exhaustion in atherosclerosis, upregulation of the co-inhibitory PD-1 expression was observed in atherosclerosis patients *(91)*. On the other hand, plaque cells could inhibit CD8 T cell activation, e.g. PD-L1 was found upregulated on macrophages in human lesions *(92)*, which could provide a co-inhibitory signal to plaque CD8 T cells. In line with impaired CD8 T cell activation in the atherosclerotic environment ApoB100 peptide vaccination did not impact cellular content of the plaques, and did not affect plaque size and stability. As vaccination with ApoB100 derived CD8 T cell epitopes did induce CD8 T cell responses but did not affect atherosclerosis, vaccination with ApoB100 derived CD8 T cell epitopes does not seem to be a viable way of treating atherosclerosis. Moreover, from a safety perspective, inducing strong CD8 T cell responses towards endogenously expressed proteins might lead to autoimmunity and tissue damage in organs where the protein is endogenously expressed *(93)*. Therefore, induction of specific tolerance towards plaque antigens or reducing overall

inflammation are likely to sort better results than inducing antigen specific CD8 T cell responses for treatment of atherosclerosis.

Although currently no drugs aimed at general reduction of inflammation are approved for treatment of cardiovascular disease by the FDA and EMA, the LODOCO *(94)* and CANTOS *(14, 95)* trial have indicated that a reduction in cardiovascular risk can be achieved through immune inhibition in humans. As immunoproteasomal inhibition with ONX-0914, inhibiting immunoproteasomal catalytic subunit LMP7 and LMP2 *(96)*, ameliorated multiple auto-immune diseases in experimental auto-immune models *(96–104)*, we assessed the effect of ONX-0914 treatment on atherosclerosis in **chapter 6**. LDLr$^{-/-}$ were fed a WTD from 15 weeks of age for 7 weeks, while fed a WTD were ONX-0914 or vehicle treated. ONX-0914 treatment reduced cDC content in the spleen and reduced cDC content and activation in mesenteric lymph nodes and cervical lymph nodes, which drain the peritoneal cavity and intestines (atherosclerosis unrelated) *(105)* and drain the aorta and supra-aortic arteries (atherosclerosis related) *(106)* respectively. Furthermore, in several lymphoid organs decreased antigen experienced effector memory (Tem) and central memory (Tcm) CD4 and CD8 T cells were observed. As the enhanced activation of DCs proofed atherogenic *(107)*, and enhanced levels of circulating Tem cells correlated with carotid intima-media thickness and circulating Tem cells were increased in patients with stable angina and acute myocardial infarction *(105)*, it is likely that ONX-0914 induced alteration in cDC and T cell populations aided in the observed atherosclerosis reduction in the ONX-0914 treated group.

Besides developing atherosclerosis, LDLr$^{-/-}$ mice on WTD develop obesity, obesity associated metabolic syndrome, and insulin resistance *(108, 109)*. Next to ameliorating atherosclerosis, ONX-0914 reduced body weight in multiple experiments with WTD fed LDLr$^{-/-}$ mice and APOE*3-Leiden.CETP mice. White adipose tissue (WAT) mass was markedly reduced in all studies, while EchoMRI in APOE*3-Leiden.CETP mice showed that lean mass was unaffected by ONX-0914 treatment. Coincident with reduced WAT mass, improved metabolic parameters such as lowered insulin levels, lowered fasting blood glucose, lowered TG levels, and in APOE*3-Leiden.CETP mice also lowered cholesterol levels were observed. To our knowledge this is the first study to show that immunoproteasomal inhibition using the LMP7 and LMP2 specific inhibitor ONX -0914 has metabolic effects in addition to its immunomodulatory effects. This is likely the linked to the use of a WTD in our study, leading to obesity and development of metabolic syndrome, instead of using lean mice on chow diet in other studies applying ONX-0914 *(96–104)*. In addition, disease-related weight loss due to induction of e.g. colitis *(99)*, EAE *(98)* or arthritis *(97)*, may have masked the metabolic effects of ONX-0914. To make sure that reduced weight (gain) in ONX-0914 treated mice was not due to (hepato)toxicity in our studies, we measured the activity of ALAT and ASAT liver enzymes in the blood, and the expression of Cyp3A11 in the liver. No indications for toxic side-effects of ONX-0914 treatment were measured.

Metabolic cage measurements with APOE*3-Leiden.CETP mice did not reveal reduced food intake or increased energy expenditure upon ONX-0914 treatment. In an oral lipid loading test using LDLr$^{-/-}$ mice, the rise in TG levels after olive oil administration was blunted in the ONX-0914 treated group. Because clearance of cholesterol and triglycerides from the blood was not affected in ONX-0914 treated mice, as assessed by injection of VLDL like particles containing radiolabeled cholesteryl oleate and glycerol trioleate in APOE*3-Leiden.CETP mice, results of the oral lipid loading test indicated reduced intestinal lipid uptake by ONX-0914 treatment. Similarly, reduced intestinal lipid uptake was observed in LMP7$^{-/-}$ animals *(110)*. Reduced intestinal lipid uptake in LMP7$^{-/-}$ mice was dedicated to reduced pancreatic lipases Pnlip and Pnlrp2 expression *(110)*, however we did not observe this upon ONX-0914 treatment. We found that ONX-0914 treatment repeatedly induced neutrophil and macrophage accumulation in white adipose tissue, likely through the upregulation of CCL2 expression we observed in mature adipocytes which were also found to express immunoproteasomal catalytic subunits. The increased levels of neutrophils and macrophages in gWAT were likely responsible for the enhanced IL-1β and TNF-α levels observed in ONX-0914 treated mice. Interestingly, patients with a loss of function mutation in PSMB8, coding for LMP7, present themselves with similar symptoms including white adipose tissue inflammation, and lipodystrophy *(111)*.

Peritoneal macrophages (including macrophages residing in gWAT) appeared to be involved in ONX-0914 mediated reduction in intestinal lipid uptake, as clodronate liposome mediated depletion of peritoneal macrophages prior to ONX-0914 treatment led to normalization of increase in TG levels upon oral lipid loading. Therefore, we looked into literature for macrophage derived factors which were reported to induce weight loss, and identified GDF15 *(112)*. GDF15 was indeed increased in the blood by ONX-0914 treatment, however also GDF15$^{-/-}$ mice lost weight upon ONX-0914 treatment, indicating that GDF15 was not mediating ONX-0914 induced weight loss. Another macrophage derived factor that has been reported to cause weight loss and reduce intestinal lipid uptake is IL-1β *(113, 114)*. Interestingly, a common side effect listed on the on the information leaflet of the (atheroprotective) IL-1β neutralizing antibody canacinumab (Ilaris) is weight gain. Therefore, it would be interesting to investigate whether IL-1β mediates ONX-0914 induced weight loss.

Treatment of mice with ONX-0914 reduces atherosclerosis and considerably reduces WAT mass in obese mice fed a WTD, concomitantly improving parameters of metabolic syndrome. Because atherosclerosis is still the primary cause of death, and the obesity epidemic is feeding metabolic syndrome related diseases worldwide, immunoproteasomal inhibition could be a valuable therapeutic tool for the western world to combat both. Currently phase 1b trials in SLE patients with the immunoproteaomal KZR-616 are ongoing, with phase 2 clinical trials focused on treatment of lupus nephritis, dermatomyositis, polymyositis, autoimmune hemolytic anemia, and immune thrombocytopenia, on the docket. It would be interesting to see whether immunoproteasomal inhibition also leads to weight loss in human, and whether there are indications for reduced cardiovascular mortality.

Future perspectives

The recent success of the CANTOS trial, reducing major cardiovascular events through administration of a neutralizing monoclonal antibody against the pro-inflammatory cytokine IL-1β (Canakinumab) *(14–16)*, implies that modulation of the immune system is also a feasible way of treating atherosclerosis and reducing cardiovascular risk in human. In line with the paradigm that lowering inflammation can reduce atherosclerosis and prevent major cardiovascular events, a clinical trial with the immunosuppressant colchicine *(18)* is still underway, while the immunosuppressive methotrexate treatment failed to reduce atherosclerosis *(19)*. When successful in preventing major cardiovascular events, colchicine could become the first immunomodulatory treatment approved for use in atherosclerosis. Dependent on the success of clinical studies with immunoproteasomal inhibitors in the context of other auto-immune diseases, immunoproteasomal inhibition in clinical trials in context of atherosclerosis and obesity could be considered. Drawbacks of general immunosuppressants are obviously increased risk for infectious diseases *(115, 116)*, but also other side effects like gastrointestinal issues, have been reported for methotrexate and colchicine which could reduce patient compliance with drug intake.

Far less side effects are to be expected with atheroprotective vaccination approaches specifically targeting plaque antigens, and especially restoring tolerance to plaque antigens. Restoring immune tolerance to plaque antigens has been effective in treating atherosclerosis in pre-clinical atherosclerosis models *(21, 117, 118)*, however has not been studied in clinical trials. In multiple sclerosis, tolerance induction towards myelin peptides in multiple sclerosis (MS) patients resulted in a decrease in antigen-specific T cell responses in a phase 1 trial *(119)*, suggesting that tolerance induction is feasible to modulate T cell mediated autoimmunity. Currently our knowledge about the antigen specific T cell responses taking place in atherosclerosis is very limited, but is known to include CD4 T cell responses directed against LDL *(38)*, collagen type V *(35)*, and HSPs *(29)*. In **chapter 3** we were able to reduce atherosclerosis by oral administration of oxLDL, but could not achieve atheroprotection in **chapter 4** through oral administration of the p210 peptide derived from the ApoB100 protein which is present in LDL, likely due to lack of a CD4 epitope in p210. Apart from difficulties identifying suitable CD4 T cell epitopes for tolerization, variations in human MHC alleles are not likely to allow a single epitope to bind all MHC-II molecules and could thereby only beneficial in some patients. Therefore, induction of tolerance towards complete antigens, or a selection of multiple CD4 epitopes should be considered for tolerization. Moreover, Induction of tolerance towards a combination of plaque antigens against which auto-immunity is developed would likely sort better effects than targeting a single antigen, targeting more T cell clones. Better characterization of the adaptive immune response, identifying antigens and antigenic epitopes in antigens could therefore be very beneficial for the treatment of atherosclerosis. Another major hurdle for application of tolerance induction in human, is the development of most effective, and safe tolerization regimens.

Induction of CD8 T cell responses towards p210 has been reported to reduce atherosclerosis *(24, 53)*, however we did not observe protective effects of vaccination with p210 in **chapter 4** or strong CD8 epitopes derived from ApoB100 in **chapter 5**. Also, when successful at inducing an atheroprotective CD8 T cell response through targeting an endogenous plaque antigen, this would likely lead to auto-immunity related side effects through killing of cells expressing the antigen outside the plaque. Due to the high risk of side effects, the use of therapeutic induction of CD8 T cell responses against plaque antigens for treatment of atherosclerosis are likely limited from a safety perspective. Still vaccination approaches in experimental settings could provide very useful insights on the role of CD8 T cells in atherosclerosis.

Besides modulation of T cell responses, induction of antibodies (IgG) to several plaque antigens, has been capable of reducing atherosclerosis in pre-clinical studies. Also induction of antibodies against (epitopes) of (ox)LDL have been studied and appeared to be atheroprotective in multiple studies *(23)*, including antibodies directed against p210 *(27)*, and were correlated with atheroprotection *(63–65)* We could not confirm atheroprotective properties of p210 antibodies in **chapter 4,** probably due to utilization of $LDLr^{-/-}$ mice instead of $ApoE^{-/-}$ mice in our study, as p210 is part of a LDLr binding site in ApoB100 *(55)*. Besides (ox)LDL antibodies, antibodies against collagen type VI have been reported to act atheroprotective *(120)*. Mechanistically, antibodies were found to inhibit macrophage activation, and enhance expression of cholesterol efflux genes through binding to FC receptors *(23, 27, 120, 121)*. Therefore, induction of antibodies against plaque antigens could be desired to treat atherosclerosis, however pathogenic activation of CD4 T cells should be limited. Through mucosal administration or use of antibody epitope mimotopes for vaccination, protective antibodies could be induced while preventing induction of proatherogenic CD4 T cells *(62)*.

There are several viable approaches through which modulation of the immune system could be deployed to reduce atherosclerosis. General immune suppression in the form of low-dose methotrexate and colchicine are currently being tested in clinical trials for treatment of atherosclerosis and if successful will likely comprise the first immunomodulatory treatments to be available for treatment of atherosclerosis *(17, 18)*. Antigen specific modulation of the immune response for treatment of atherosclerosis is further away, however pre-clinical studies have indicated that such approaches are feasible for treatment of atherosclerosis. A combination of lipid and immune management, and promoting a healthy lifestyle will likely comprise the preventive measures of the future to combat atherosclerosis and cardiovascular disease.

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