Immunity in atherosclerosis: novel assays, biomarkers and therapeutic approaches
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Atherosclerosis is a complex chronic disease, in which the immune system play a critical role. As is outlined in the introduction (*Chapter 1*), cells of our immune system can be eitheratherogenic or atheroprotective, depending on the specific cell subset investigated. Modulation of the immune system during development and progression of atherosclerosis in mouse models has been shown to be effective in decreasing plaque size or increasing plaque stability. The first successful clinical trials have been performed, where immunomodulation reduced the chance of recurrent cardiovascular events in cardiovascular disease patients.[1,2] The CANTOS trial, using the anti-IL-1β antibody canakinumab, showed that canakinumab treatment in cardiovascular disease (CVD) patients with high sensitivity (hs)CRP levels >2mg/L reduced the risk for recurrent cardiovascular events compared to placebo.[3] However, these patients also had a higher risk for fatal infections. Furthermore, colchicine treatment reduced the risk for recurrent cardiovascular events compared to placebo.[1,2] Interestingly, while colchicine has a broad immunosuppressive function, it also inhibits the inflammasome, and thereby IL-1β production, showing that inhibition of the inflammasome is a viable method to modulate atherosclerosis. However, given the adverse effects found in the CANTOS trial, further research is necessary to elucidate the optimal strategy for immunomodulation in atherosclerotic disease. Furthermore, assays to study immunomodulatory drug effects in clinical trials are necessary to assess efficacy of these drugs.

The research in this thesis aimed to identify potential endpoints for clinical trials related to atherosclerotic disease and related potential drug targets, and describes the development of assays to study drug effects in clinical trials. Furthermore, novel strategies for immunomodulation of atherosclerosis are investigated, both in mice and in human. The research performed is also summarized in Figure 1.

I. Identification of potential biomarkers of atherosclerotic disease, and analytical development of target engagement assays for future atherosclerosis-targeted immunomodulatory drugs

In the first part of this thesis, the focus was to identify potential biomarkers that may serve as endpoints for early phase clinical pharmacology studies and develop assays to test drug functionality that can be used in these clinical trials. In *Chapter 2* the effect of two risk factors for atherosclerosis development, ageing and smoking, and the effect of cardiovascular disease

on the human immune system was investigated to identify potential cellular subsets that can be used as clinical biomarker. In order to study the effects of these risk factors, five groups of volunteers were included in a observational clinical trial: young healthy volunteers (18–25 years), elderly healthy volunteers (>60 years), young smokers (18–25 years), heavy smokers (>45 years) and patients with stable coronary artery disease (>60 years). Immune cell subsets were analyzed by flow cytometry, and circulating protein levels were measured by ELISA and proteomics. Furthermore, monocyte and T cell responses were analyzed using whole blood stimulation assays. Although the groups were rather small (n=30 for YH, n =20 for EH, n=20 for YS, n=11 for HS and n=27 for CAD), clear differences could be observed between the groups. Firstly, clear ageing effects were observed, which corroborated with effects of immunosenescence known from literature.[4,5] Senescent CD4+CD28null and CD8+CD57+ T cells were increased in elderly healthy volunteers and patients with CAD. Previous studies have shown that a high frequency of senescent CD28null and CD57+ T cells in peripheral blood and/or atherosclerotic plaques strongly associate with hyperglycaemia, acute cardiovascular events and mortality.[6-8] Secondly, clear smoking effects were observed, as illustrated by an increase in central memory T cells and an increase in TH1 cells with corresponding IFNγ producing T cells. These effects were also known from literature.[9] Interestingly, a significantly lower level of circulating OXLDL-specific IgM was found in the patients with stable CAD. Furthermore, lower circulating PC-specific IgM levels were found in both the CAD patients and healthy elderly volunteers. Both these types of antibodies play an atheroprotective role by facilitating the clearance of apoptotic cells[10,11] and preventing foam cell formation by blocking OXLDL uptake by macrophages.[12] Lastly, proteomics analysis was performed to measure circulating inflammatory markers, showing an increase in plasma TREM1 and CCL11 in elderly volunteers compared to young volunteers. Elevated IL-6 plasma levels were found in heavy smokers and patients with CAD. It has been shown that increased IL-6 plasma levels correlate with risk for CAD.[13-15] Lowering of IL-6 in the CANTOS trial correlated with a decrease risk of recurrent cardiovascular events[16] and furthermore, a clinical trial with IL-6 inhibitor ziltivekimab in patients with high atherosclerotic risk is currently ongoing (ZEUS trial), establishing the relevance of this cytokine as marker of disease. In*Chapter 3*, the effect of LPS on neutrophils was evaluated. Neutrophils have been suggested to play a role during

the onset of atherosclerosis, but they are also implied in plaque destabilization.[17-19] Currently, no treatments specifically targeting neutrophils have been registered, although this could be a valuable method to prevent cardiovascular events by stabilizing atherosclerotic plaques. To be able to test novel drugs targeting neutrophils in human, stimulation of neutrophils is required, since neutrophils are not in an activated state in basal conditions. Short-term inflammation can be induced in a human endotoxemia model, by intravenous LPS administration. In addition, whole blood *in vitro* cultures with LPS stimulation can be a useful assay to use in pre-clinical development or as target engagement assay in early clinical studies. Neutrophil activation by LPS in healthy volunteers was compared between an *in vitro* (whole blood cultures) and an *in vivo* (human endotoxemia) challenge. Activation markers CD11b, CD62L, CD63 and CD64 were measured by flow cytometry. The expression of CD11b, CD63 and CD64 was increased after exposure to LPS, while CD62L expression decreased, both *in vitro* as well as *in vivo*. Furthermore, levels of granule proteins myeloperoxidase, neutrophil elastase and LL37 were increased after LPS exposure, both *in vitro* in culture supernatant and *in vivo* in circulation, showing an increase in neutrophil degranulation after LPS stimulation. Lastly, little differences were found in the proteome of neutrophils after *in vivo* LPS exposure, which can be explained by the fact that neutrophils already have all necessary proteins stored in their granules. In general, activation of neutrophils by LPS *in vivo* and *in vitro* resulted in similar neutrophil activation. Based on these results, *in vitro* and *in vivo* LPS challenges can be used in future clinical studies evaluating neutrophil-targeted investigational compounds. As is illustrated by the CANTOS trial, inhibition of IL-1β results in a decrease in recurrent cardiovascular events in CVD patients with hsCRP >2 mg/L.[3] Testing the effects of inflammasome inhibitors requires *in vitro* and *ex vivo* assays mimicking the *in vivo* milieu as much as possible. Therefore we set-up a whole blood based assay using lipopolysaccharide (LPS) and ATP as inflammasome activators (*Chapter 4*). We measured the time-frame of IL-1β and IL-18 secretion upon stimulation, thereby demonstrated that NLRP3 is activated in these settings. We here also show that monocytes and neutrophils are the main contributors to the measured IL-1β secretion. To further elucidate the optimal conditions for performing whole blood cultures, we performed additional experiments, described in *Chapter 5*. In this chapter we show that the age of the whole blood sample has a strong effect on the

amount of cytokine production after LPS stimulation, while the amount of cytokine secreting cells remain stable during sample aging. Cytokine production after LPS stimulation can mainly be attributed to monocytes.[20] Sample ageing also resulted in a moderately diminished cytokine production after whole blood PMA/ionomycin and SEB stimulation, which is mainly T cell driven. Addition of cell culture medium, which restores nutrients present in the culture, did not prevent the decline in cytokine production with sample ageing. These data together show that standardization of the time after blood sampling is important for consistent results during a clinical trial.

II. Immunomodulation of atherosclerosis

Part II of this thesis focuses on testing of immunomodulatory compounds in humans or in LDLR$^{-/-}$ mice. The four chapters included in this section all describe the use of immunomodulatory compounds that could potentially be developed or used for additional treatment for atherosclerosis. First, a registered vaccine and a drug were tested in humans. In *Chapter 6*, the effect of vaccination with the pneumococcal vaccine Prevenar-13 on the circulating levels of OXLDL-specific antibodies was investigated. In mice, vaccination with *Streptococcus pneumoniae* resulted in an increase in atheroprotective OXLDL-specific IgM levels, and a corresponding decrease in atherosclerosis development,[21] indicative of a the atheroprotective potential for humans as well. However, vaccination with Prevenar-13 in human did not result in an increase of OXLDL-specific antibodies. Certain IgM clones binding OXLDL also bind phosphorylcholine (PC), present in the cell wall of *Streptococcus pneumoniae*.[12,21] The PC content in Prevenar-13 is low, which could explain the absence of the desired vaccination effects in humans. Potentially, a vaccine developed to elicit a response against PC would be successful in increasing circulating OXLDL antibody levels in humans, as it does in mice.[22] In *Chapter 7* research on hydroxychloroquine (HCQ) is described. HCQ is a broadly acting immunosuppressive drugs, originally developed for the treatment of malaria. HCQ is also prescribed for auto-immune diseases such as rheumatoid arthritis and systemic lupus erythematosus. The OXI trial, investigating HCQ for prevention of recurrent cardiovascular events in myocardial infarction patients, is currently ongoing.[23] However, the exact working mechanism of HCQ is unknown, and little data is available on the translatability of *in vitro* and *in vivo* effects. Therefore we

performed *in vitro* experiments using PBMCs studying the effect of HCQ on endosomal TLR-mediated cytokine responses as well as on T and B cell proliferation. We performed the same assays with PBMCs from healthy volunteers who received multiple doses of HCQ. The concentrations where effects were seen in *in vitro* experiments were not reached in *in vivo* plasma samples. However, since HCQ accumulates intracellular in the lysosome, it could be that local concentrations of HCQ are in fact higher, and that therefore HCQ does modulate cellular responses. Lastly, the effect of inhibition of the co-stimulatory signal for T cell activation is described in this thesis. *Chapter 8* describes the first-in-human clinical trial using KY1005 (currently named amlitelimab), an OX40L inhibitor. Inhibition of OX40L should result in a decrease in T cell activation upon stimulation, which was studied in this clinical trial using vaccination with the neo-antigen keyhole limpet hemocyanin (KLH) and tetanus toxoid as a recall antigen. KY1005 did not significantly impact the humoral response to KLH or TT. However, the KLH recall response, induced by an intradermal KLH injection 3 weeks after vaccination, was significantly suppressed in the KY1005-treated groups compared to placebo. Currently, no OX40-OX40L inhibitor is on the market, however the use of such a drug in patients with cardiovascular disease caused by atherosclerosis would be very interesting to investigate since inhibition of the OX40-OX40L pathway in mice significantly reduced plaque size,[24] and even regression of established atherosclerotic plaques.[25] B cells express OX40L upon activation, with which they interact with activated T cells. Moreover, follicular helper T cells express OX40. These cells are essential for B cell help by inducing class-switching of B cells amongst other functions.[26] Inhibition of the OX40-OX40L pathway prevents class-switching of B cells in mice.[27] In our clinical trial, we did not observe significant effects of OX40L blockade on KLH-specific IgG and IgM responses. However, when looking at the KLH-specific IgM/IgG ratio at the highest tested dose of KY1005 (12 mg/mL), a potential drug-dependent inhibition of class-switching was observed (IgM/IgG ratio of 0.34 ± 0.17 for placebo vs 0.56 ± 0.13 for 12 mg/mL KY1005), hinting that inhibition of class-switching also occurs in humans. In *Chapter 9* the effect of inhibition of T cell activation by stimulation of the co-inhibitory PD-1 pathway is reported. LDLR^{-/-} mice were treated with an agonistic PD-1 antibody during administration of a western-type diet (WTD) to induce atherosclerosis development. We showed that stimulation of the PD-1 pathway decreased atherosclerosis development. Furthermore, a decrease

in CD4^{+} T cells in the plaque was found upon PD-1 stimulation. PD-1 stimulation resulted in a decrease in IFNγ-producing T cells, while the amount of IL-10 producing T cells was increased in the spleen. An increase in regulatory B cells, B1 cells and associated circulating OXLDL-specific IgM levels was found as well. Lastly, stimulation of the PD-1 pathway delayed WTD-induced monocyte increase in circulation up to 3 weeks. These data show that stimulation of the co-inhibitory PD-1/PD-L1/2 pathway decreases atherosclerosis development in mice. Therefore, next to inhibition of co-stimulatory pathways, stimulation of co-inhibitory pathways could be potentially useful therapies to treat atherosclerosis in humans.

Figure 1 Summary of the studies described in this thesis.
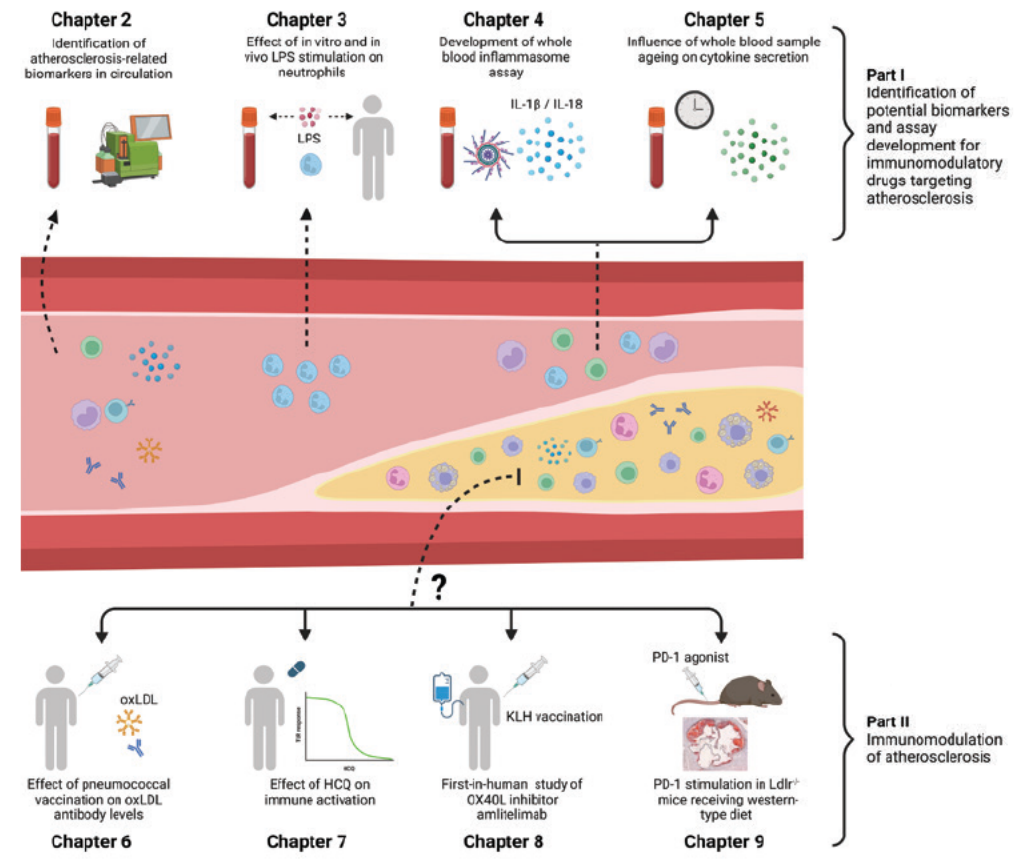
FUTURE PERSPECTIVES

Treatment of atherosclerosis currently mainly consist of lifestyle changes and a regimen lipid-lowering drugs with other drugs such as beta-blockers and anti-coagulants. This treatment is successful in preventing recurrent cardiovascular events in a subpopulation of CVD patients. However, recurrent cardiovascular events remain a high risk for CVD patients.[28] For these patients at high risk, immunomodulation could be a useful additional therapeutic strategy. To predict which patients are at risk for a recurrent event and would benefit from additional treatment, reliable diagnostic biomarkers are necessary. Sub-analysis of the CANTOS trial showed that the efficacy of canakinumab treatment in reducing secondary cardiovascular events was greater in patients where canakinumab treatment reduced hsCRP levels to <2 mg/L compared to patients where hsCRP levels remained >2 mg/L.[29] Furthermore, patients who achieved IL-6 levels below the study median had a 52% reduction in cardiovascular mortality while patients receiving canakinumab with IL-6 levels above study median had no significant reduction.[16] These data indicate that hsCRP and IL-6 could be biomarkers used as an indication for the clinical effectiveness of immunomodulatory therapy. However, all patients included in the CANTOS trial had an increased level of hsCRP, and not all patients benefited from treatment. In the future, immunomodulatory atherosclerosis treatment will become more personalized: patients with a low basal inflammatory state probably do not benefit as much as patients with a high basal inflammatory from immunomodulatory treatment. A combination of biomarkers, in addition to the known markers such as total cholesterol and LDL, could possibly be used to create a 'risk profile' for CVD patients, and guide subsequent treatment.

While immunomodulation of atherosclerosis is currently mainly focusing on the prevention of recurrent cardiovascular events, prevention of a primary cardiovascular event would of course be ideal. Since atherosclerosis is a slowly developing and chronic disease, immunomodulatory therapies would have to be applied over a prolonged period of time. Therefore, treatment would have to be highly specific and with minimal impact on overall immune function. The two drugs shown to be successful in prevention of recurrent cardiovascular events, canakinumab and colchicine, are both broadly immunosuppressive. The same is true for hydroxychloroquine, also described in this thesis. For this reason, these drugs are probably not the most suitable approaches for prevention of atherosclerosis development. Inhibition of co-stimulatory pathways or stimulation of co-inhibitory pathways are both more specific in the sense that they target mainly T cell function. However, suppressing general T cell function over longer periods of time probably also increases infection risk. A highly specific method for atherosclerosis modulation, with theoretically limited to no expected adverse effects, would be the induction of OXLDL-specific IgM. Unfortunately, this could not be achieved with Prevenar-13 vaccination in human, although the concept has been shown to be successful in mice (*Chapter 6*). A newly developed vaccine that induces OXLDL-specific IgM production would be beneficial. However, the development of such a vaccine will be challenging, since it should mainly elicit a IgM response and preferably no IgG response, since OXLDL-specific IgG is considered atherogenic.[30,31] This could possibly be obtained by using a vaccine that targets B1 cells and not B2 cells, in a T-cell independent manner. Furthermore, inhibition of the OX40-OX40L pathway during vaccination to prevent class-switching of B2 cells could also be a way to mainly induce a IgM response. It should be investigated if class-switching does not occur at a later stage upon recognition of the OXLDL antigen however. Other ways to induce tolerance during atherosclerosis development should also be investigated, for example the induction of regulatory T cells that react against OXLDL-specific T and/or B cells would be beneficial to prevent atherosclerosis development or progression.

CONCLUSIONS

Prevention of atherosclerosis progression and destabilization of the plaque is necessary to prevent (recurrent) cardiovascular events, for which immunomodulation is a promising strategy. The research described in this thesis can add to the development of such a therapy. In part I, potential biomarkers and the development of whole blood based assays are described for use in future clinical trials using immunomodulatory drugs. These assays and biomarkers can help to assess the functionality of these drugs. Part II describes novel strategies for immunomodulation in atherosclerosis both in humans and mice. While currently no immunomodulatory therapies exist for atherosclerosis treatment, this will probably become available in the not-too-distant future.

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