Immunity in atherosclerosis: novel assays, biomarkers and therapeutic approaches

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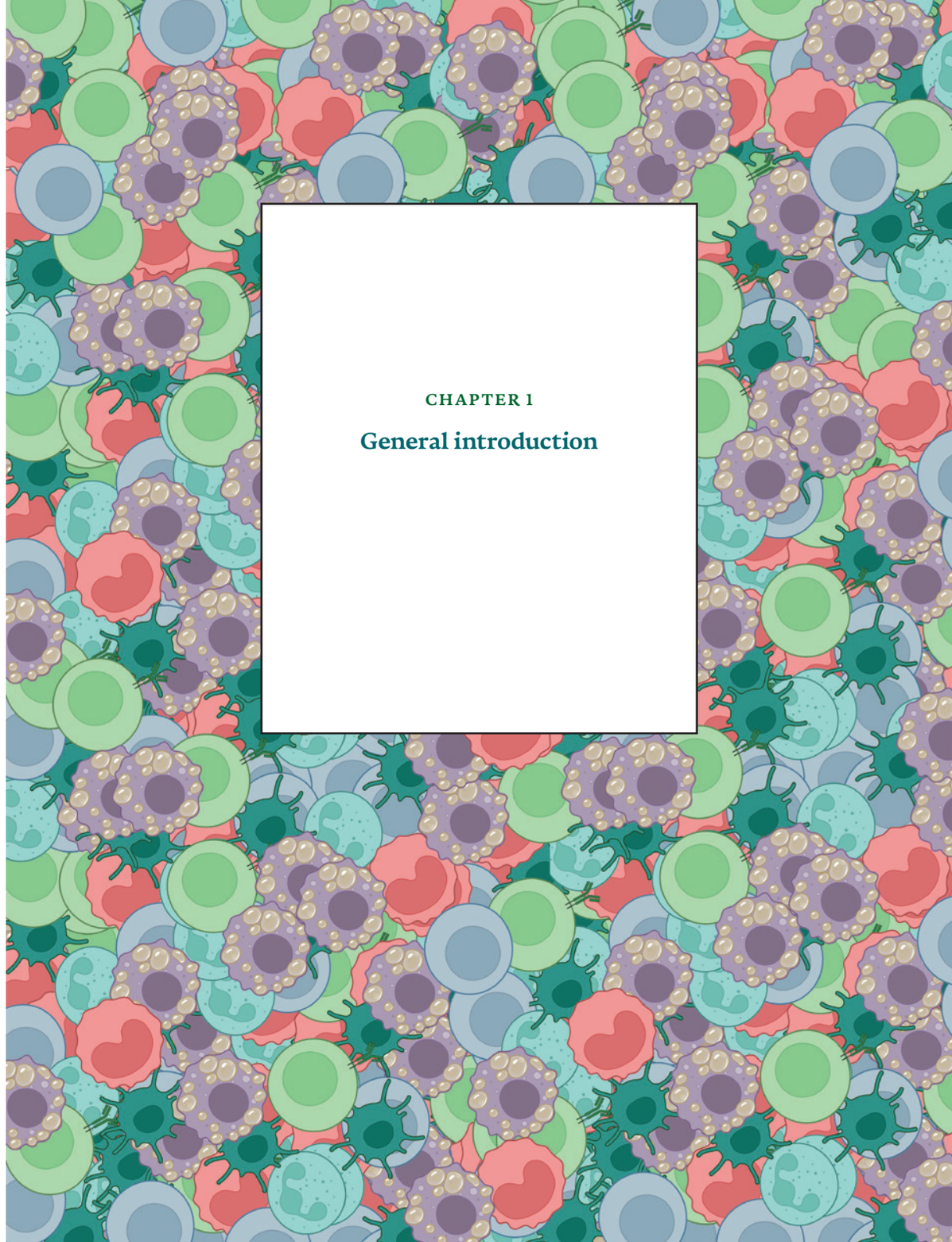


CHAPTER 1

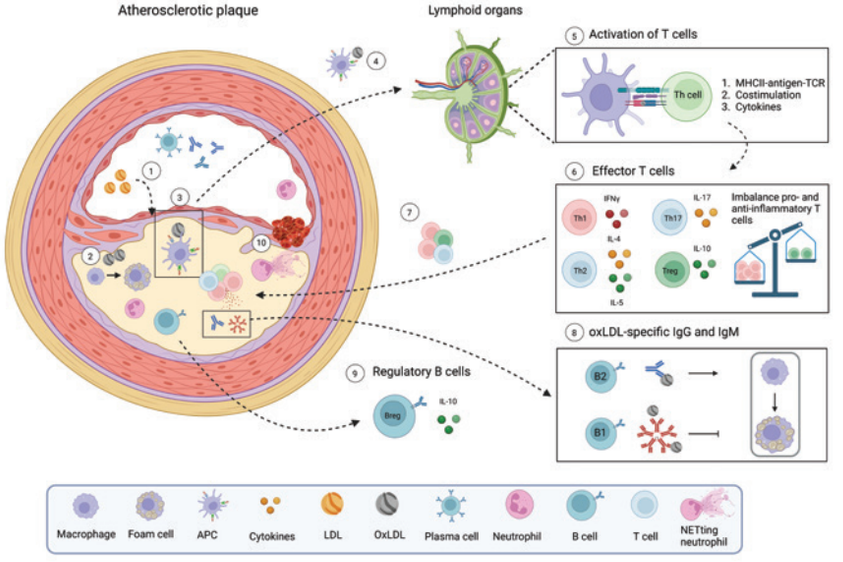
General introduction

Introduction

Cardiovascular disease (CVD) is the main cause of death worldwide.[1] CVD includes many diseases, including stroke, myocardial infarction, heart failure, heart rhythm disorders and congenital heart disease.[1] The main underlying pathology of many CVDs is atherosclerosis. Atherosclerosis is characterized by the development of an atherosclerotic plaque, or lesion, in the arterial wall. Ultimately, atherosclerosis development can lead to atherothrombosis, which in turn can lead to cardiac arrest, pulmonary embolism or stroke and eventually death.

The onset of atherosclerosis development starts already in adolescents.[2-4] While atherosclerosis gradually develops in many people, several risk factors are known that enhance the risk for atherosclerosis development. Some are modifiable and mostly lifestyle related, such as smoking, excessive alcohol use and diet, while other risk factors unmodifiable, such as age and genetic predisposition. Development of atherosclerosis generally starts with a disbalance in serum cholesterol levels, mainly an increase in low density lipoprotein (LDL). While LDL is atherogenic, high density lipoprotein (HDL) is inversely associated with atherosclerosis development, likely due to extraction of cholesterol from tissues and transport to the liver where it is excreted.[5-7] LDL accumulates in the subendothelial space in the vessel wall, at sites of endothelial dysfunction and flow perturbation, where it undergoes oxidation. Currently, the main treatment to prevent acute cardiovascular syndromes aims at lowering cholesterol levels, in addition to lifestyle modifications, anti-thrombotic drugs and blood pressure lowering drugs. Although atherosclerosis development is indeed primarily lipid-driven, the immune system plays a critical role during the onset and progression of atherosclerotic plaques. Therefore, atherosclerosis is currently seen as a chronic inflammatory disease. While treatment with lipid-lowering drugs is, at least partly, successful, recurrent cardiovascular events remain a high risk for CVD patients,[8] showing a need for additional treatment. In addition, elucidation of the role of the immune system during atherosclerosis development and in the plaque micro-environment is essential for development of new diagnostic tools and treatments for CVD.

Figure 1 Immune cell subsets during the onset and progression of atherosclerosis. Low density lipoprotein (LDL) migrates into the vessel wall where it is oxidized (OXLDL) (1).Macrophages phagocytose OXLDL and differentiate into foam cells (2). Other immune cells are attracted to the site of inflammation. Antigen-presenting cells, such as dendritic cells, phagocytose OXLDL (3), and subsequently migrate to the secondary lymphoid organs (4). Here, OXLDL epitopes are presented via MHC class II molecules to T helper cells which in turn get activated (5) and clonally expanded (6). Skewing to T helper cell subsets is dependent on the microenvironment.TH1 cells are known to be atherogenic via the production of IFNγ, while IL-5 (produced by TH2 cells) and IL-10 (produced by regulatory T cells) are atheroprotective. The role of IL-4 and IL-17 in this process remains inconclusive. There is an imbalance between pro- and anti-inflammatory T cells during atherosclerosis development. Next, activated T cells migrate to the plaque (7). B cells can play both an atherogenic as well as an atheroprotective role via the production of immunoglobulins (8). IgG (produced by B2 cells) binds OXLDL and thereby facilitates the uptake of OXLDL by macrophages. In contrast, when IgM (produced by B1 cells) binds OXLDL the uptake is inhibited and clearance is promoted. Regulatory B cells play an atheroprotective role via the production of IL-10 (9). Neutrophils play an atherogenic role, both during the onset as well as during the destabilization of the advanced plaque, mainly by NETOSIS which destabilizes the plaque, which may lead to atherothrombosis (10).



IMMUNE CELL SUBSETS IN ATHEROSCLEROSIS

The immune system plays an important role in atherosclerosis. Upon accumulation of oxidized LDL (OXLDL) in the vessel wall, OXLDL is taken up via scavenger receptors by macrophages,[9] after which these macrophages differentiate into lipid laden foam cells[9,10]contributing to inflammation in the vessel wall. Besides LDL and OXLDL, cholesterol crystals can accumulate in macrophages, resulting in inflammasome activation[11,12]which can further enhance inflammation. In addition to macrophages, neutrophils migrate to the lesion site in an early stage. Upon activation, neutrophils induce expression of adhesion molecules, adding to the endothelial dysfunction, and release myeloperoxidase (MPO), which facilitates reactive oxygen species formation and contributes to the oxidation of LDL[13]. While the innate immune system plays an important role in the early stages of plaque development, during atherosclerosis progression the adaptive immune system is also involved. T cells accumulate in the plaque, of which some clones recognize OXLDL-specific epitopes,[14,15] contributing to the inflammatory response. The role of T cells in atherosclerosis development is complex, with some subsets playing an atherogenic role while others are atheroprotective. The same is true for B cells, B1 cells generally are atheroprotective via the production of autoantibodies against oxidized LDL, while other B cell subsets promote atherosclerosis development.

The section below describes the role of monocytes and macrophages, neutrophils, T cells and B cells in atherosclerosis. While other immune cells, including but not limited to dendritic cells, NK cells, and mast cells also influence atherosclerosis development, the role of these cells will not be discussed in this thesis.

Monocytes and macrophages

As mentioned in the previous section, monocytes play a critical role in atherosclerosis development. In human, three major subsets are identified based on CD14 and CD16 expression. Classical monocytes ($CD14^{+}$) are the largest group, followed by non-classical monocytes ($CD14^{dim}CD16^{+}$) and intermediate monocytes ($CD14^{+}CD16^{+}$).[16] Classical monocytes are mainly involved in phagocytosis, a process in which large (pathogenic) particles or apoptotic cells are ingested and degraded internally.[17] These particles or dead cells are recognized by amongst others scavenger receptors. Classical monocytes secrete high quantities of pro-inflammatory cytokines and chemokines and infiltrate inflamed tissues. They express higher levels of chemokine receptors compared to the other subsets.[18,19] Non-classical monocytes produce lower levels of pro-inflammatory cytokines and chemokines compared to classical monocytes[20] and, in circulation, they promote neutrophil adhesion to the vessel wall via TNFα secretion.[21,22] Intermediate monocytes are considered to be 'inflammatory', as is illustrated by the increase in intermediate monocytes in patients with systemic infections.[23,24] A large proportion of studies investigating atherosclerosis is performed using mouse models. In mice, two main subsets in monocytes are identified based on LY6C expression, $LY6C^{hi}$ inflammatory monocytes, and $LY6C^{int}$ patrolling monocytes. $LY6C^{hi}$ monocytes correspond to classical/intermediate monocytes in human, while $LY6C^{int}$ monocytes correspond to non-classical monocytes.

Hypercholesterolemia results in an increase in circulating monocyte levels and their activation status.[25,26] During the onset of atherosclerosis, monocytes are attracted to the subendothelial space in response to endothelial dysfunction and upon infiltration in the arterial wall, these monocytes differentiate into macrophages. As mentioned before, macrophages play a critical role during the onset of atherosclerosis by phagocytosis of lipids. Indeed, the absence of macrophages in $APOE^{-/-}$ mice with an M-CSF mutation results in a decrease in atherosclerosis development.[27] Furthermore, selective knockout of monocytes and macrophages in CD11b-diphtheria toxin receptor transgenic mice showed decreased atherosclerosis development.[28]Activation of the inflammasome via cholesterol crystals or OXLDL leads to inflammasome formation and subsequent cell death via pyroptosis which is a highly inflammatory process and results in further activation of the innate immune system. Inflammasome activation is necessary for the production and release of pro-inflammatory cytokines interleukin (IL)-1β and IL-18, that play an important role during atherosclerosis development.[29]

Like monocytes, macrophages can be divided into multiple subsets. Non-differentiated or naïve MO-macrophages can differentiate into multiple subsets dependent on the microenvironment. Despite the existence of more subsets, and the high plasticity and ability of macrophages to repolarize, the main subsets are M1-like and M2-like macrophages, which can be discriminated via gene signatures and protein expression.[30] In general,

M2-like macrophages are considered anti-inflammatory, while M1-like macrophages are pro-inflammatory. M1 macrophages play a significant role in plaque progression,[31] via secretion of high levels of pro-inflammatory cytokines and by maintaining chronic inflammation. Furthermore, M1 macrophages are the pre-dominant macrophage subtype in murine as well as human lesions and are associated with progressing plaques, while M2-like macrophages are associated with atherosclerotic plaque regression, most likely via promoting macrophage plaque egression and resolving inflammation.[32,33]

Neutrophils

Neutrophils are the most abundant type of leukocytes in human blood. They are the first responders upon an infection, and have short life-spans of approximately 5 days. Neutrophils contain granules, which can be released upon activation, containing proteolytic enzymes such as myeloperoxidase (MPO), neutrophil elastase, ROS and anti-microbial peptides. Release of some of these granules already occurs upon endothelial binding and entrance of extravascular tissue. Neutrophils can eventually undergo apoptosis upon activation, which is tightly regulated to prevent tissue damage.[34] A unique form of cell death specific for neutrophils is NETosis, via the formation of neutrophil extracellular traps (NETs). NETosis can be induced by several stimuli such as pathogens, cytokines, microcrystals, antibodies and immune complexes.[35] Induction of NETosis is dependent on NADPH oxidase activation and subsequent ROS formation. Upon activation, chromatin decondensation occurs and this is accompanied by a disturbance in the plasma membrane ultimately resulting in the formation of a NET, which captures pathogens. However, in contrast to apoptosis, NETosis leads to an increased inflammatory response and can lead to tissue damage. In human, neutrophils can be identified by surface markers CD16, CD66b and CD15. In mice, LY6G can be used to identify neutrophils.

Similar to monocytes, hyperlipidemia causes an increase in circulating neutrophil levels.[36,37] Neutrophils are known to contribute during later stages of atherosclerosis, notably in plaque destabilization and atherothrombosis, as is illustrated by several studies in human plaques showing that neutrophils are present in unstable rupture-prone plaques, but not in stable plaques.[38-40] However, more recently the role of neutrophils in the early stages of atherosclerosis development has gained interest. Neutrophils for example accumulate in the vessel wall in LDLR$^{-/-}$ mice on a Western-type diet.[41] It has been shown that neutrophil depletion using a LY6G antibody in APOE$^{-/-}$ mice significantly decreased plaque size after 4 weeks of treatment, but not in established plaques after 16 and 52 weeks of plaque development.[37] Furthermore, neutrophils induce recruitment of monocytes to inflamed areas, such as via the release of the cathelicidin LL37, or the murine homolog CRAMP. Furthermore, binding of LL37 to RNA or DNA, including self-DNA released by for example NETosis, results in increased type-I interferon (IFN) responses,[42,43] resulting in an enhanced inflammatory response. Indeed, CRAMP$^{-/-}$ APOE$^{-/-}$ mice displayed a reduced plaque size with low macrophage numbers as compared to APOE$^{-/-}$ mice.[44]

T cells

T cells develop from the common lymphoid progenitors in the bone marrow, after which they migrate to the thymus where they mature. During maturation in the thymus, T cells start to express a T cell receptor (TCR) that recognizes short peptides presented via a major histocompatibility complex (MHC) molecule.[45] Thereafter, T cells undergo selection by epithelial cells expressing high numbers of MHC-I and MHC-II molecules.[46] T cells that bind strongly to the MHC molecules undergo apoptosis, since they are highly likely to be self-reactive. T cells that do not respond at all undergo delayed apoptosis,[46,47] while T cells that moderately respond receive survival signals. After maturation and selection, naïve T cells express a unique T cell receptor and leave the thymus to migrate into circulation.

Upon recognition of an antigen via the MHC–TCR interaction, which is the first signal of T cell activation, T cells require a second signal for full activation via so-called co-stimulatory molecules of which many belong to the B7 superfamily.[48] The third signal of T cell activation, via cytokines secreted by antigen-presenting cells (APC), results in full activation and skewing of T cells. Besides co-stimulatory molecules, co-inhibitory molecules are expressed on T cells and APCs. These co-inhibitory molecules function in a similar manner as co-stimulatory molecules however dampen T cell activation.[49] The expression of both co-stimulatory and co-inhibitory molecules regulates T cell activation. The role of some of these co-stimulatory and co-inhibitory pathways in atherosclerosis development is displayed in table I. In general, inhibition of co-stimulatory and activation of co-inhibitory pathways are protective for atherosclerosis development.

Table 1 Co-stimulatory and co-inhibitory molecules and their role in atherosclerosis.

Co-stimulatory pathways		Refs
CD40–CD40L	Inhibition results in decrease in atherosclerosis development and more stable plaques.	[50-53]
OX40–OX40L	Inhibition results in decreased atherosclerosis development and regression of plaques.	[54-56]
CD28–CD80/CD86	CD28 is constitutively expressed on T cells on most T cells, and absence of CD28 leads to anergic T cells. CD80/CD86 deficiency reduces atherosclerosis development. CTLA4 shares the same ligands as CD28.	[57]
CD27–CD70	The CD27-CD70 pathway is essential for B cell proliferation and Ig synthesis. Stimulation of CD70 is atheroprotective, CD70 deficiency leads to increased plaque formation.	[58, 59]
CD30–CD30L	Inhibition results in reduced atherosclerosis development.	[60]
Co-inhibitory pathways		
PD-1–PD-L1/PD-L2	Knockout of PD-1 or PD-L1/PD-L2 results in decreased atherosclerosis development.	[61-63]
CTLA4–CD80/CD86	CTLA4 blockade enhances atherosclerosis development. Overexpression of CTLA4 decreased atherosclerosis development.	[64-66]
BTLA–HVEM	Stimulation of BTLA protected against atherosclerosis.	[67]

T cells can be divided into two main subsets, the CD4$^+$ T helper cells (TH), and CD8$^+$ cytotoxic T cells (TC), which can be divided into several subsets.

CD8$^+$ cytotoxic T cells

CD8$^+$ T cells recognize antigen presented via major histocompatibility complex (MHC)-I molecules, which are expressed on all nucleated cells. Cells usually present cytosolic self-antigen in MHC-I molecules, which does not induce an immune response. However, upon infection, pathogen-specific antigen is presented, which is recognized by CD8$^+$ T cells. Upon recognition of an antigen, and a secondary stimulatory signal, CD8$^+$ T cells kill the target cell expressing the recognized antigen via the release of perforin and granzymes, binding of Fas ligand to Fas receptor on the target cell and via production of cytokines.

The role of CD8$^+$ T cells in atherosclerosis is controversial, as both pro- as well as anti-atherogenic effects have been described. The presence of CD8$^+$ T cells in the human plaque has first been shown in the late 1980's by immunostaining,[68,69] which has during the years been confirmed with additional techniques, such as single cell RNA sequencing.[70,71] It has been shown that hypercholesterolemia in APOE$^{-/-}$ mice increased the number of IFNγ expressing CD8$^+$CD28$^+$ T cells,[72] and another study showed that CD8$^+$ T cells control monopoiesis and decreased circulating monocyte levels, thereby contributing to plaque macrophage burden.[73] Furthermore, CD8$^+$ T cells promote the development of vulnerable plaques by inducing apoptosis of macrophages, endothelial and smooth muscle cells, andvia secretion of perforin and granzyme B leading to necrotic core formation.[74] On the other hand, several studies have shown atheroprotective effects of CD8$^+$ T cell subsets. For example, immunization with an apoB100 peptide resulted in an expansion of CD8$^+$ T cells and a reduction of plaque development.[75] Furthermore, regulatory CD8$^+$CD25$^+$ T cells reduce atherosclerosis development in APOE$^{-/-}$ mice.[76] These data show that different CD8$^+$ T cell subsets may play opposing roles in atherosclerosis development.

CD4$^+$ T helper cells

CD4$^+$ T helper cells, as the name indicates, 'help' activate CD8$^+$ T cells and/or B cells during an inflammatory response. They recognize antigens presented via major histocompatibility complex (MHC)-II molecules, which are expressed on APCs, but can be upregulated on other cells upon stimulation. Upon recognition of their antigen and subsequent activation, naïve CD4$^+$ T cells, also named TH0 cells, undergo clonal expansion and differentiate into effector T cell subtypes. Dependent on the microenvironment, mainly controlled by the cytokines secreted by APCs, these naïve T cells differentiate into TH1, TH2, TH9, TH17, TH22 or regulatory T cells. TH1, TH2, TH17 and regulatory T cells and their role in atherosclerosis development are described further below and summarized in Table II.

TH1 CELLS Differentiation of TH0 cells into TH1 cells is mainly induced by IFNγ and IL-12.[77-79] TH1 cells are characterized by expression of transcription factor T-BET, and they are the main producers of IFNγ.[80] TH1 cells play an essential role in the defense against intracellular pathogens including viruses and intracellular bacteria. Dysregulation of TH1 cells has been associated with several autoimmune diseases, such as rheumatoid arthritis[81] and systemic lupus erythematosus.[82] In atherosclerosis, TH1 cells have been found to play a pro-atherogenic role. A study using T-BET deficient LDLR$^{-/-}$ mice showed that atherosclerosis development was significantly reduced in these mice compared to LDLR$^{-/-}$ mice.[83] Moreover, multiple studies have shown a pro-atherogenic role for the TH1 hallmark cytokine IFNγ.

APOE$^{-/-}$IFNγR$^{-/-}$ mice develop substantially less atherosclerotic plaque compared to APOE$^{-/-}$ mice.[84] Furthermore, administration of exogenous IFNγ enhances atherosclerosis in APOE$^{-/-}$ mice,[85] confirming the atherogenic role of IFNγ in atherosclerosis.

TH2 CELLS IL-4 is the main driver of differentiation of TH0 cells into TH2 cells.[86,87] TH2 cells express transcription factor GATA3,[88] and participate in the defense against extracellular parasites and are also involved in allergic reactions after encountering an allergen. Amongst other cytokines, TH2 cells are characterized by their IL-4, IL-5 and IL-13 production, cytokines that contribute to eosinophil activation and promote antibody production by B cells.[89] In contrast to TH1 cells, the role of TH2 cells in atherosclerosis is inconclusive. IL-4 counteracts the production of IFNγ,[90] which would be beneficial in the case of atherosclerosis development. However, IL-4 deficiency decreased plaque development in LDLR$^{-/-}$ mice,[91] although another study failed to show any effects of either administration of IL-4 or IL-4 deficiency on atherosclerosis development in APOE$^{-/-}$ mice.[92] IL-13 deficiency in LDLR$^{-/-}$ mice enhanced atherosclerosis, while IL-13 administration in mice with established lesions favorably altered plaque composition by induction of M2 macrophages.[93] IL-5 produced by TH2 cells stimulates B1 cells and subsequent OXLDL-specific IgM production.[94] In human, it has been shown that high circulating numbers of TH2 cells is associated with a decreased mean common carotid intima-media thickness and a reduced risk of acute myocardial infarction in women.[95] These data show that the role of TH2 cells in atherosclerosis is still inconclusive, and likely dependent on specific cytokine secretion.

TH17 CELLS The cytokines transforming growth factor (TGF)-β and IL-6 drive differentiation of TH0 cells to TH17 cells,[96] which then start to express transcription factor RORC (RORγT in mice).[97] TH17 cells are characterized by their production of IL-17 and, like TH2 cells, TH17 cells play a role in protection from extracellular pathogens. Like TH1 cells, dysregulation of TH17 cells is associated with autoimmunity.[98-100] The role of TH17 cells, and IL-17, in atherosclerosis is controversial. Blockade of IL-17 by administration of an IL-17 blocking antibody in APOE$^{-/-}$ mice resulted in a decrease in atherosclerosis development.[101,102] However, an increase in IL-4 levels and a decrease in IFNγ was observed, suggesting that a decreased TH1 response was in part responsible for the reduction in atherosclerosis.[102] Other studies show that IL-17 blockade via an IL-17 antibody did not affect plaque formation in either LDLR$^{-/-}$ or APOE$^{-/-}$ mice.[103] Furthermore, studies using IL-17 or IL-17R knockout mice display conflicting results. Two studies using APOE$^{-/-}$IL-17$^{-/-}$ mice show a decrease in atherosclerosis development by reducing the amount of infiltrated macrophages in the plaque and aorta,[104,105] while other studies show no effect or even an increase in atherosclerosis development.[106,107]

REGULATORY T CELLS Regulatory T cells (TREGS) are a subset of CD4$^+$ T cells specialized in the suppression of the immune response, thereby mediating inflammation and ensuring self-tolerance. TREGS exert their immunosuppressive function via secretion of anti-inflammatory cytokines such as IL-10, IL-35 and TGF-β, but also via direct suppression of activated lymphocytes via binding of co-inhibitory molecules. TREGS are characterized by their expression of transcription factor forkhead box P3 (FOXP3). TH cells differentiate into TREGS under the influence of IL-2 and TGF-β.[108-110] TREGS play a protective role in atherosclerosis due to their immunosuppressive function. Deletion of TREGS via knockout of co-stimulatory factors CD80 and CD86, both necessary for TREG development, resulted in an increase in atherosclerosis development.[111] Later, more TREG specific studies have been performed confirming these findings, both vaccination against FOXP3, and elimination of FOXP3$^+$ TREGS aggravated atherosclerosis development.[112,113] Administration of IL-2/anti-IL-2 complexes potently induced TREGS and thereby suppressed atherosclerosis development.[114]

While T helper cell differentiation is dependent on the micro-environment, T helper cell differentiation is not final, and T helper cells show plasticity. For example, during inflammatory conditions, such as atherosclerosis TREGS can lose FOXP3 expression and become pro-inflammatory TH cells.[115] Indeed, APOB100 specific T cells have been shown to be able to switch their phenotype to either a mixed phenotype, or re-differentiate into another subset completely.[116] TREGS and TH17 cells are most prone to change their phenotype in the atherosclerotic plaque, during which TREGS lose their immunosuppressive function. This plasticity makes research into the role of T cells during atherosclerosis development, and potential therapeutic T cell targets more difficult.

Table 2 Summary of Th subsets

T helper cell subtype	Cytokines driving differentiation	Role in immune defense	Production of cytokines
TH1	IFNγ, IL-12	Intracellular pathogens, viruses, intracellular bacteria	IFNγ
TH2	IL-4, IL-2	Extracellular pathogens, parasites	IL-4, IL-5, IL-13
TH17	TGF-β, IL-6	Extracellular pathogens, fungi	IL-17A, IL-17F, IL-21, IL-22
TREG	TGF-β, IL-2	Anti-inflammatory	IL-10, IL-35, TGF-β

B cells

B cells are mainly known for their function in antibody secretion. Antibodies consist of two identical fragment antigen binding (FAB) domains and a fragment crystallizable (FC) domain connected via a hinge region.[117] The FAB domains are specific for each antibody and are created via somatic DNA rearrangement leading to a vast amount of different antibodies that can be produced.[118] Based on the FC domain, 5 antibody isotypes can be identified, immunoglobulin (IG)A, IgD, IgE, IgG and IgM. IgD, IgE and IgG exist as monomers, while IGA forms a dimer and IgM forms a pentamer complex. Binding of an antibody to an antigen can block the function of the antigen, while the antigen is also opsonized for uptake by immune cells via FC receptors.[119] Besides antibody production, B cells also function as antigen presenting cells via MHC-II molecules[120] and are able to produce pro- and anti-inflammatory cytokines. B cells can be categorized into subpopulations of which B1 cells, B2 cells and regulatory B cells are described below.

B2 CELLS After development from progenitor cells in the bone marrow, immature B2 cells, expressing surface IgM, mature in circulation and secondary lymphoid organs, such as the spleen and lymph nodes. These immature or transitional B cells differentiate further into marginal zone (MZ) or follicular (FO) B cells. FO B cells are the largest B cell subset and are characterized through IgD expression and populate the B cell follicles in the spleen or lymph nodes. Upon encountering an antigen and T cell help via co-stimulatory receptors and/or cytokines, FO B cells either differentiate into short-lived plasma cells, or enter the germinal center. In the germinal center, these cells proliferate and differentiate into memory B cells or long-lived plasma cells. B cells undergo class-switching under the influence of T follicular helper (TFH) cells. The micro-environment, involving mainly cytokines produced by T cells, influences class-switching. TH2 cells are potent inducers of IgG1 and IgE class-switching, while TH1 cells induce a IgG2 response.[121-123] MZ B cells are mainly involved in T cell-independent responses, during which they quickly produce large amounts of IgM.[124-126] As their name implies, MZ cells can mostly be found in the marginal zone of the spleen.

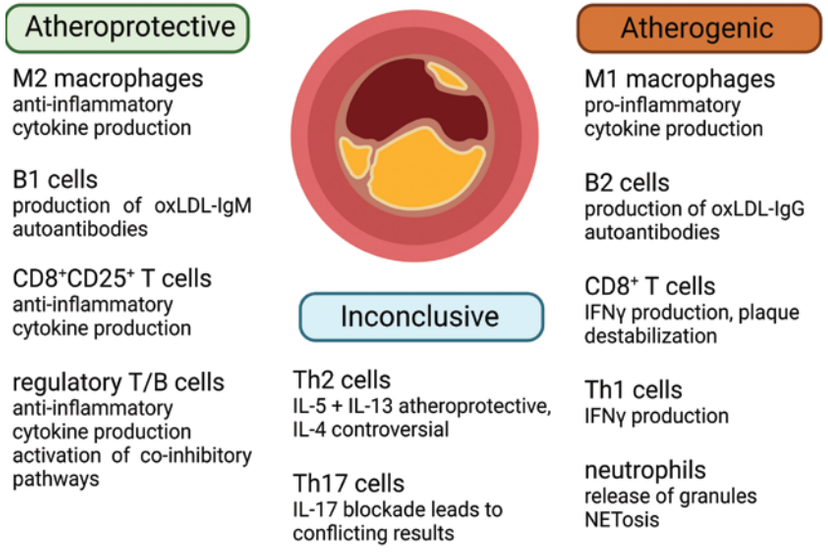
B1 CELLS The B1 cell comprises a small B cell subset that spontaneously produces IgM, mostly directed at self-antigen.[127] Like MZ cells, B1 cells can quickly produce IgM in a T cell independent manner.[125,126] Since B1 cells are involved in T cell independent and innate-like immune responses, they are sometimes considered as part of the innate immune system instead of the adaptive immune system. B1 cells are the most abundant B cell population in the peritoneum and pleural cavities, and are present in low amounts in the spleen and bone marrow.[127] B1 cells are mainly developed during fetal development, de novo development of B1 cells during adulthood is restricted.[128,129] B1 cells are maintained by 'self-renewal'.

B cells are known to play a dual role in atherosclerosis, depending on their subset. Depletion of B cells results in a decrease in atherosclerosis development,[130] indicating an atherogenic role for B cells. However, adoptive transfer of MZ B cells[131] and B1 cells[132] is shown to be atheroprotective. Indeed, depletion of B2 cells, but not B1 cells resulted in a decrease in atherosclerosis development,[133] confirming an atherogenic role for B2 cells. The main reason B1 cells have an atheroprotective function is via the production of natural IgM antibodies against for example OXLDL. OXLDL IgM is atheroprotective by blocking uptake of OXLDL by macrophages, and by facilitating the clearance of apoptotic cells, thereby decreasing atherosclerotic plaque formation.[134-139]

REGULATORY B CELLS Furthermore, recently the role of anti-inflammatory regulatory B cells (BREGS) has gained interest.[140] BREGS can arise from multiple B cell subsets at different stages of B cell development, suggesting that the micro-environment influences BREG development rather than specific lineage factors.[141] There are multiple ways via which BREGS exert their anti-inflammatory function, such as via secretion of anti-inflammatory cytokines IL-10[142] and IL-35,[143] or via surface proteins such as immune checkpoint inhibitor PD-L1,[144] FAS ligand[145] or ectoenzyme CD73.[146] Indeed, there

are studies that show that BREGS reduce atherosclerosis development.[147] In other studies no effect of Bregs on plaque size was observed,[148-149] however, a reduction in total circulating leukocytes, inflammatory monocytes and CD4 T cell activation was observed, with an increase in IL-10$^+$ CD4$^+$ T cells, all atheroprotective effects.[149] It should be noted that all these studies investigated IL-10$^+$ BREGS, the role of BREGS that function via surface proteins has not been widely investigated in atherosclerosis yet. Recently, TIM-1 was found to be a marker for BREGS,[150,151] and it has been shown that mice with a deficiency in TIM-1 signalling, show an increase in atherosclerosis development compared to wild type mice.[152]

Figure 2 Summary of immune cell effects in atherosclerosis. M2 macrophages, B1 cells, CD8$^+$CD25$^+$ T cells and regulatory T and B cells play atheroprotective roles during the progression of atherosclerosis. The role of TH2 cells and TH17 cells remains inconclusive, with some studies showing a protective effect, while others show an atherogenic effect. M1 macrophages, B2 cells, CD8$^+$ T cells, TH1 cells and neutrophils play atherogenic roles during atherosclerosis development and progression. Supporting references are included in the body text.



IMMUNOMODULATION OF HUMAN ATHEROSCLEROSIS

The effects of immunosuppression on (recurrent) cardiovascular events have recently been studied in human. Several clinical trials were performed in CVD patients, or patients at risk for CVD.

IL-1β has been shown to be an important pro-inflammatory mediator during atherosclerosis development. Inhibition of IL-1β or the inflammasome, which upon activation results in IL-1β release, decreases atherosclerosis development in mice.[153,154] Furthermore, IL-1β activates other cells, among which smooth muscle cells, to produce IL-6.[155] The CANTOS trial has shown for the first time that immunosuppression, in this case using the anti-IL-1β monoclonal antibody canakinumab, reduces the risk for recurrent cardiovascular events in patients with high hsCRP levels.[156]

It was hypothesized by the investigators of the CANTOS trial, that reduction of hsCRP and/or IL-6 play critical roles in the effectiveness of immunosuppression in the prevention of recurrent cardiovascular events. Further analysis of the CANTOS trial showed that patients treated with canakinumab, with reduced levels of IL-6, had a reduced risk for major adverse cardiovascular events of 32%, and a 52% reduced risk for cardiovascular mortality.[157] The same was observed for hsCRP: when canakinumab treatment lowered hsCRP below 2 mg/L, cardiovascular mortality risk was reduced by 31%.[158] Furthermore, hsCRP levels are predictive of risk of myocardial infarction[159] and hsCRP levels >3 mg/L were associated with incidence of major adverse cardiovascular events (MACE) in patients who underwent coronary angiography for acute coronary syndrome or stable angina pectoris.[160] However, inhibition of IL-1β resulted in an increase in fatal infections,[156] showing that the balance of immune activation and immunosuppression has to be carefully navigated.

Other successful trials are the LODOCO trials in which colchicine treatment was given. Administration of colchicine to either patients who had a myocardial infarction within 30 days prior to admission to the study,[161] or in patients with chronic coronary disease[162,163] showed a decrease in cardiovascular death, MI, stroke or urgent hospitalization compared to the placebo group. Colchicine has a broad immunosuppressive function, which mainly acts on the innate immune system. It suppresses neutrophil adhesion and recruitment and inhibits the NLRP3 inflammasome in macrophages.[164]

While IL-6 and hsCRP levels were not measured in the LODOCO trials, it is possible that colchicine also reduces IL-1β levels, and subsequently IL-6, via its inhibitory effect on the inflammasome. A study investigating the effect of the IL-6 inhibitor ziltivekimab in patients with high atherosclerotic risk (moderate to severe chronic kidney disease and hsCRP levels >2 mg/ml), showed decreased hsCRP levels compared to placebo.[165] In addition, a pilot study using hydroxychloroquine in hospitalized MI patients showed a significant reduction in IL-6 plasma levels compared to placebo, although no effect on hsCRP was found.[56,166] Both studies did not investigate the effect on cardiovascular outcomes, although currently studies investigating the effects on cardiovascular endpoints are ongoing.

Low dose methotrexate is a successful treatment for RA, however low dose methotrexate administration in patients with previous MI or multivessel coronary disease and type-2 diabetes or metabolic syndrome (CIRT trial) did not prevent recurrent cardiovascular events or reduce hsCRP or pro-inflammatory cytokine levels.[167] However, patients enrolled in the CIRT trial did not have elevated hsCRP levels, in contrast to the CANTOS trial. In addition, treatment with the MAPK inhibitor losmapimod did not prevent cardiovascular death, MI or severe recurrent ischemia.[168,169] While an initial decrease in hsCRP was seen after 72h of losmapimod treatment, this decrease did not last during the treatment time.[169]

These trials show that immunomodulation, in addition to current regular treatments, is a viable strategy to prevent recurrent cardiovascular events in patients with established atherosclerosis and increased inflammation markers. Trials are ongoing to further elucidate which type of immunomodulation is the most beneficial for atherosclerosis patients. It should be noted that no preventive clinical studies have been performed investigating the effect of immunomodulation on primary cardiovascular events.

THESIS OUTLINE

The immune system plays a critical role during the onset and progression of atherosclerosis. The first clinical trials evaluating the effects of immunomodulatory drugs on atherosclerosis show promising results. However, further research is warranted to elucidate the optimal strategy to inhibit atherosclerosis development and prevent cardiovascular events caused by atherosclerosis. The immunomodulatory drugs currently researched are broadly immunosuppressive and display adverse events related to general immune suppression. This thesis describes research investigating novel immunology-based strategies to modulate pathways relevant for atherosclerosis in both human and mice, and studies to identify and validate potential biomarkers and assays for monitoring of atherosclerosis-targeted immunotherapy in future clinical trials. The thesis comprises two parts:

1 Identification of potential biomarkers of atherosclerotic disease, and analytical development of target engagement assays for future atherosclerosis-targeted immunomodulatory drugs;
2 Clinical and preclinical testing of immunomodulatory compounds, with a potential value for atherosclerosis prevention or treatment.

Part I: Identification of potential atherosclerosis related biomarkers and development of assays for use in clinical trials.

In ***Chapter 2*** we have investigated the effects of two risk factors of atherosclerosis development, ageing and smoking, on the human immune system. Five groups of volunteers were included in this study: young healthy volunteers (18-25 year of age), elderly healthy volunteers (>60 years), young smokers (18-25 years), heavy smokers (>45 years) and patients with stable coronary artery disease (>60 years). We analyzed circulating immune cell numbers by flow cytometry, tested monocyte and T cell responses using whole blood stimulation assays, and performed proteomics to assess the levels of circulating pro-inflammatory proteins in these groups. In ***Chapter 3*** we aimed to develop suitable models driving neutrophil activation. As is mentioned above, neutrophils play a role during the onset of atherosclerosis, but are also implied in plaque destabilization. Therefore, drugs targeting neutrophils could beuseful in prevention of cardiovascular events via plaque-stabilization. Since neutrophils are not inan activated state in healthy volunteers, stimulation of neutrophils is required for evaluation of the immunomodulatory effects of neutrophil-targeted drugs. Intravenous (i.v.) administration of LPS in healthy volunteers is an established method to induce short-term inflammation. The *in vivo* effect of intravenous LPS administration on neutrophils was evaluated in healthy volunteers and compared with the effects of *in vitro* activation, in LPS-challenged whole blood cultures from healthy donors. Chapters 4 and 5 describe whole blood-based assays, often used during clinical trials to evaluate drug activity. In ***Chapter 4*** the development of a whole blood NLRP3 inflammasome assay is

described. As mentioned above, the inflammasome plays an important role during the development of atherosclerosis. Inhibition of IL-1β successfully prevented recurrent cardiovascular events in cardiovascular disease patients.[156] Future inflammasome-targeted drugs may benefit from the availability of a solid target engagement assay, which can guide early phase clinical pharmacology studies for such compounds. ***Chapter 5*** also describes the development of whole blood assays. In this chapter, the effect of the ageing of whole blood samples on immune responsivity is investigated. Whole blood samples were stimulated with LPS to activate monocytes, or PMA and ionomycin or SEB to activate lymphocytes, and the time window between blood collection and incubation start was varied up to 24 hours. Insights into the effects of sample ageing on assay performance are critical when implementing the assay in future studies to monitor drug activity.

Part II: Immunomodulation of atherosclerosis

Chapter 6 describes a clinical trial in which healthy subjects were vaccinated with the pneumococcal vaccine Prevenar-13 or placebo, to study the effects on circulating antibodies to OXLDL. In mice, immunization with *Streptococcus pneumoniae* resulted in an increase in circulating OXLDL-specific IgM levels and a subsequent decrease in atherosclerosis development, explained by molecular mimicry between OXLDL and phosphorylcholine.[170] We evaluated the clinical translation of this concept. Healthy volunteers were randomized to receive 0, 1, 2 or 3 vaccinations with Prevenar-13, and OXLDL and PC antibody levels and serum lipids were assessed from baseline up until 68 weeks after first dose. In ***Chapter 7*** we investigated immunosuppression by hydroxychloroquine (HCQ)*in vitro* and *ex vivo.* HCQ is a broad immunosuppressive drug, commonly used for treatment of multiple autoimmune diseases. HCQ is currently being evaluated in a clinical study for its effect on recurrent cardiovascular events.[171] However, the compound's exact mechanisms of action and the dose-response relationship are poorly understood. We performed immune response assays using immune cells from healthy volunteers who received oral doses of HCQ (*ex vivo* drug activity), or HCQ was added directly to the PBMC cultures (*in vitro* drug activity). We studied the effect of HCQ on endosomal TLR stimulation of PBMCs with TLR3, 7 and 9 ligands, by measuring the IFNα and IL-6 production to assess effects on both IRF and NFκB pathways. Furthermore, the effect on T and B cell activation and proliferation was assessed using Cell Trace Violet staining, and by measuring cytokine release. ***Chapter 8*** describes a clinical trial in which the immunosuppressive effect of the OX40L blocking antibody amlitelimab was evaluated on vaccination responses to a neo-antigen (keyhole limpet hemocyanin, KLH) and a recall antigen (tetanus toxoid). As has been shown before, blockade of the OX40-OX40L pathway significantly reduces atherosclerosis development.[54,56] Therefore, OX40L blockade in human could be a useful tool to prevent (recurrent) cardiovascular events. In ***Chapter 9*** we investigated the effect of stimulation of immune checkpoint PD-1 using an agonizing antibody on atherosclerosis development in $LDLR^{-/-}$ mice. Inhibition of co-inhibitory pathway PD-1 has shown to aggravate atherosclerosis development, while inhibition of co-stimulatory pathways decreases atherosclerosis. Therefore, it was hypothesized that stimulation of the PD-1/PD-L1 pathway would decrease atherosclerosis development.

Finally, the results reported in this thesis are summarized and discussed in ***Chapter 10***, in the context of current knowledge and future perspectives.

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