Immunity in atherosclerosis: novel assays, biomarkers and therapeutic approaches

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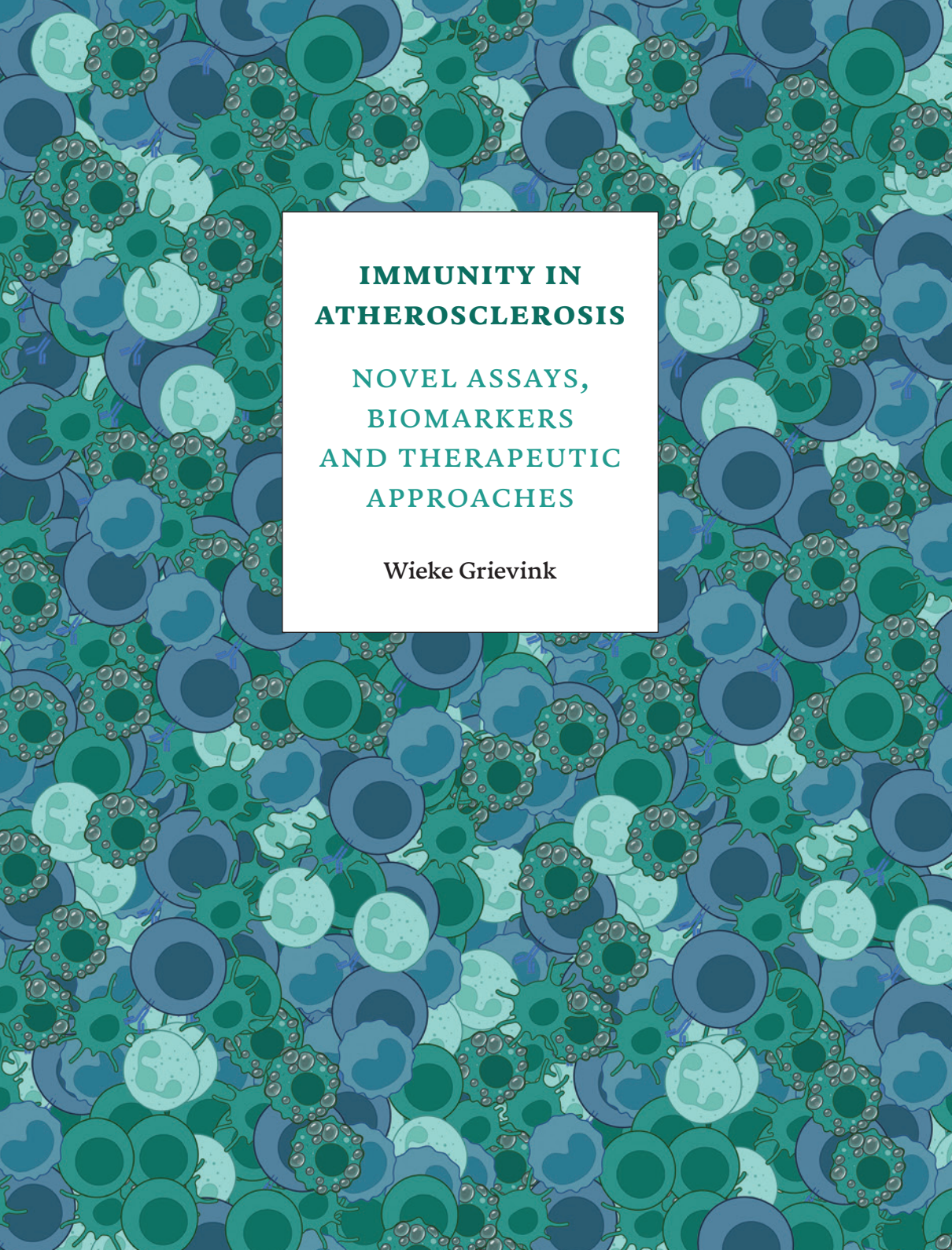

IMMUNITY IN ATHEROSCLEROSIS

NOVEL ASSAYS, BIOMARKERS AND THERAPEUTIC APPROACHES

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IMMUNITY IN ATHEROSCLEROSIS:

NOVEL ASSAYS, BIOMARKERS AND THERAPEUTIC APPROACHES

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IMMUNITY IN ATHEROSCLEROSIS

NOVEL ASSAYS, BIOMARKERS AND THERAPEUTIC APPROACHES

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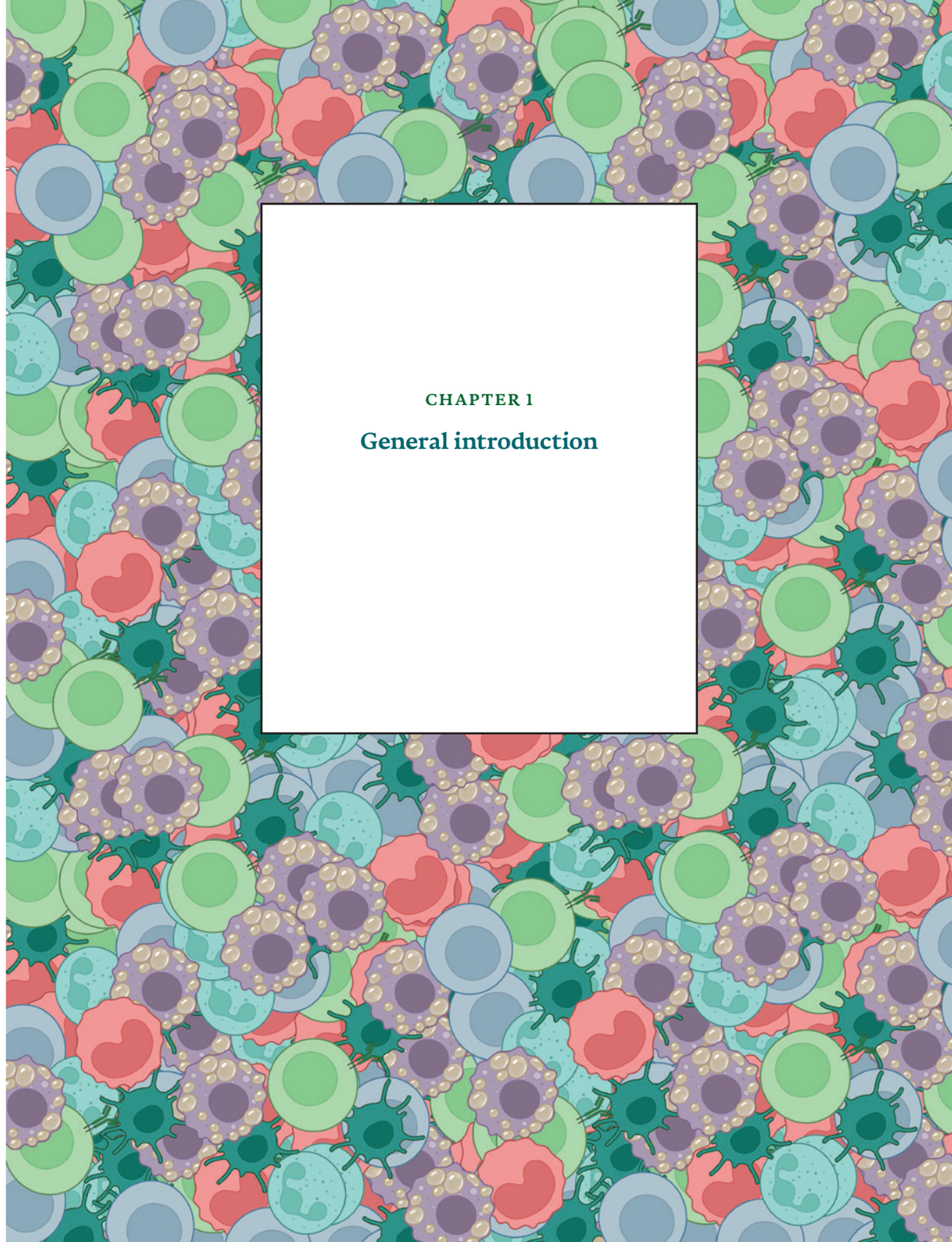


CHAPTER 1

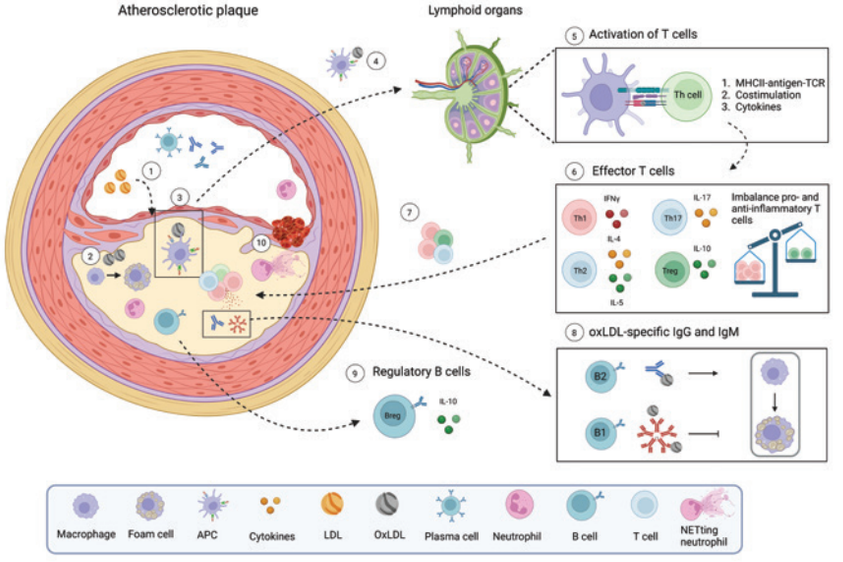
General introduction

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.

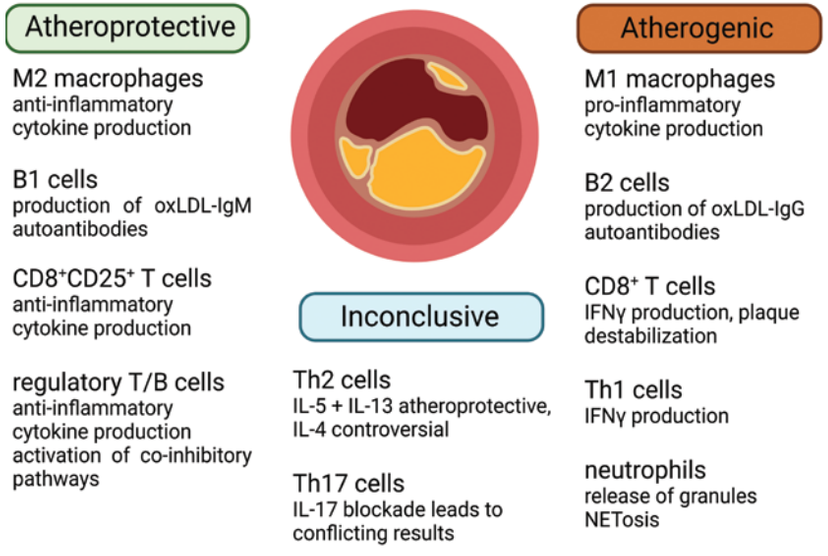
B1 CELLS The B1 cell comprises a small B cell subset that spontaneously produces IgM, mostly directed at self-antigen.[127] Like MZ cells, B1 cells can quickly produce IgM in a T cell independent manner.[125,126] Since B1 cells are involved in T cell independent and innate-like immune responses, they 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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SECTION I

IDENTIFICATION OF POTENTIAL ATHEROSCLEROSIS RELATED BIOMARKERS AND DEVELOPMENT OF ASSAYS FOR USE IN CLINICAL TRIALS

CHAPTER 2

Cardiovascular risk factors: the effects of ageing and smoking on the immune system, an observational clinical study

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Graphical abstract

Figure 8 Summary of the main differences caused by ageing, smoking and CAD.

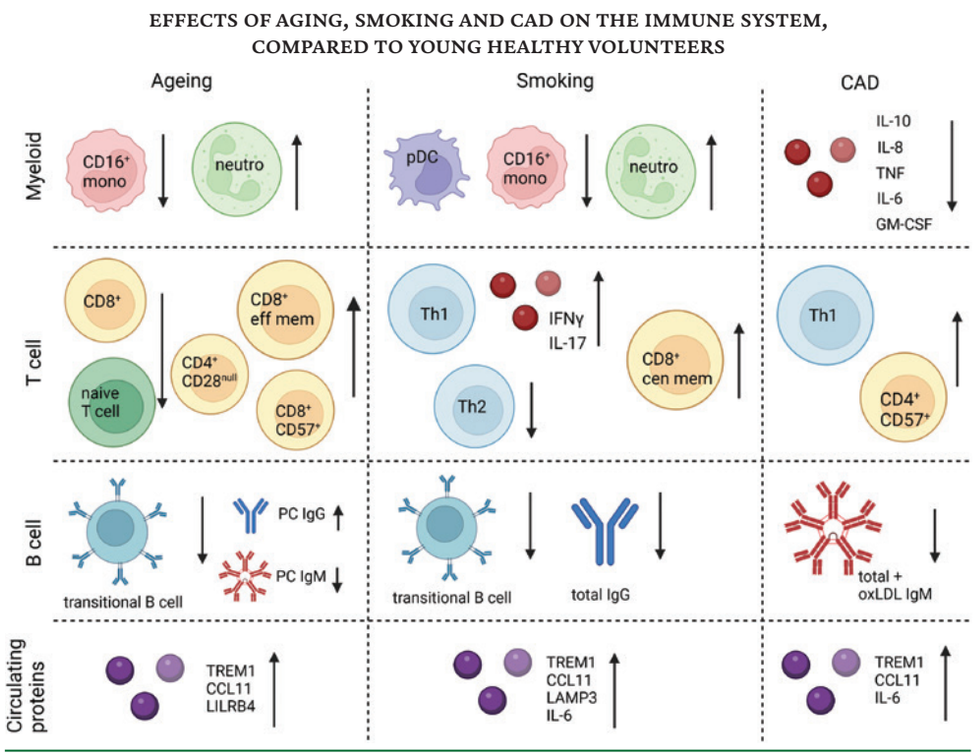


Figure is created using biorender.com.

Abstract

Currently immunomodulatory compounds are under investigation for use in patients with cardiovascular disease, caused by atherosclerosis. These trials, using recurrent cardiovascular events as endpoint, require enrollment of large patient groups. We investigated the effect of key risk factors for atherosclerosis development, ageing and smoking, on the immune system, with the objective to identify biomarkers differentiating between human populations, and potentially serving as endpoints for future phase 1B trials with immunomodulatory compounds.

Blood was collected from young healthy volunteers (aged 18-25 years, n=30), young smokers (18-25 years, n=20), elderly healthy volunteers (>60 years, n=20), heavy smokers (>45 years, 15 packyears, n=11) and patients with stable coronary artery disease (CAD) (>60 years, n=27). Circulating immune cell subsets were characterized by flow cytometry, and collected plasma was evaluated by proteomics (Olink).

Clear ageing effects were observed, mostly illustrated by a lower level in CD8$^+$ and naïve CD4$^+$ and CD8$^+$ T cells, with an increase in CD4$^+$ and CD8$^+$ effector memory T cells in elderly healthy volunteers compared to young healthy volunteers. Heavy smokers showed a more inflammatory cellular phenotype, especially a shift in TH1/TH2 ratio: higher TH1 and lower TH2 percentages compared to young healthy volunteers. A significant decrease in circulating atheroprotective OXLDL-specific IgM was found in patients with CAD compared to young healthy volunteers. Elevated pro-inflammatory and chemotactic proteins TREM1 and CCL11 were observed in elderly volunteers compared to young volunteers. In addition, heavy smokers had an increase in pro-inflammatory cytokine IL-6 and lysosomal protein LAMP3. These data show that ageing and smoking are associated with an inflammatory immunophenotype, and that heavy smokers or aged individuals may serve as potential populations for future clinical trials investigating immunomodulatory drugs targeted for cardiovascular disease.

Introduction

The main underlying cause of cardiovascular disease (CVD) is atherosclerosis. Atherosclerosis development starts with formation of oxidized low-density lipoprotein (OXLDL). OXLDL is taken up by macrophages which differentiate into foam cells in the vessel wall, leading to inflammation. During this process other immune cells are attracted to the area and eventually an atherosclerotic plaque is formed.[1] The role of the immune system in this process is complex. A vast number of immune cell subsets play an atherogenic role, such as macrophages,[2] TH1 cells[3] and B2 cells.[4] Other immune cell populations are thought to be atheroprotective, generally by suppressing the immune system. Examples are regulatory T cells via the production of anti-inflammatory cytokines such as IL-10[5], but also B1 cells that produce the anti-atherogenic OXLDL-specific IgM.[6] Binding of OXLDL-specific IgM to OXLDL leads to a decreased uptake by macrophages and an enhanced clearance by the liver.[7] The balance between pro- and anti-atherogenic cells and cytokines released by these cells is lost during atherosclerosis development.

Currently, patients that are diagnosed with CVD caused by atherosclerosis are generally treated with cholesterol-lowering drugs, beta blockers and/or anti-coagulants. While this is a successful treatment leading to a reduction in circulating LDL cholesterol, a large proportion of CVD patients remain at high risk for recurring cardiovascular events. Therefore, immunomodulation has recently gained interest as a potential therapy for atherosclerosis. In the CANTOS trial treatment with the anti-IL-1β antibody canakinumab results in fewer cardiovascular events in patients with high plasma CRP levels and previous myocardial infarction, compared to placebo.[8] Furthermore, a study investigating the effect of low-dose colchicine (LODOCO trial) on recurrent cardiovascular events, showed that colchicine treatment significantly reduces the risk of cardiovascular events.[9] However, treatment of patients with low-dose methotrexate (CIRT trial) did not show an effect on myocardial infarction, stroke or cardiovascular death.[10] Between these studies however, inclusion criteria differed, as patients in the CIRT and LODOCO trials had either type 2 diabetes or metabolic syndrome in addition to previous myocardial infarction, while patients in the CANTOS trial had enhanced hsCRP levels in addition to previous myocardial infarction.

The aforementioned trials required large study groups and a long follow-up time to assess the functionality of the investigated immunomodulatory drug. Early phase clinical trials for an initial assessment of the safety, pharmacokinetics and pharmacodynamics of novel therapeutic drug candidates are however commonly conducted in relatively small groups of healthy volunteers. Evaluation of drug activity in healthy volunteers can be challenging, especially for immunomodulatory drugs, as generally, immune activation is lacking in healthy subjects. For such drugs, early single or multiple ascending dose programs in healthy volunteers are commonly enriched with *ex vivo* cell stimulation assays or *in vivo* immune challenges to evaluate drug effects. Alternatively, early inclusion of a small group of subjects with differentiating immune endpoints or patients can be considered: in a phase 1B setting, the effect of one or a few drug doses on specific immune endpoints could provide insight into the dose-activity relationship.

Because of the immunological basis underlying the pathophysiology of atherosclerosis, and the potential value of well-characterized populations with an altered immune system/response for early phase clinical pharmacology trials, we characterized immune system parameters in small groups of volunteers, stratified for age, smoking behavior, and disease. We investigated the effect of ageing and smoking, known risk factors for atherosclerosis development, on circulating immune cell phenotype, immune cell functionality, and circulating inflammatory protein levels. We included young healthy volunteers (aged 18–25 years), elderly healthy volunteers (>60 years), young smokers (18–25 years), and heavy smokers (>45 years) in the study, and also enrolled stable coronary artery disease (CAD) patients (>60 years) as reference population. We aimed to identify immune endpoints that clearly differentiated between volunteer groups, so that ultimately these populations could serve as 'disease model' to run small phase 1B studies with immunomodulators under development for cardiovascular disease, thereby circumventing the use of patients with CAD in these trials due to possibly interfering medication use by these patients.

Materials and Methods

SUBJECTS

In total 108 male subjects were enrolled between April 2019 and March 2020 (Figure 1). The study took place at the Centre of Human Drug Research in Leiden, the Netherlands. The study was approved by the Independent

Ethics Committee of the Foundation "Evaluation of Ethics in Biomedical Research" (Stichting Beoordeling Ethiek Biomedisch Onderzoek), Assen, the Netherlands and Declaration of Helsinki principles were followed. The study is registered in the Dutch Trial Register (Nederlands Trial Register (NTR)) under number NL7754.All subjects signed an informed consent form prior to any study-related activity.

Five groups of subjects were included: young healthy volunteers (YH) aged between 18 and 25, elderly healthy volunteers (EH) aged >60 years, young smokers (YS) aged 18-25 years, smoking 8 cigarettes/day for at least 6 months, heavy smokers (HS) aged >45 years smoking at least 15 pack years and stable coronary artery disease patients (CAD) aged >60 years. The CAD group was considered stable after undergoing a revascularization procedure and being without recurrent events for at least 1 year. Subjects were not allowed to take any medication, except for the CAD group. Subjects underwent a general medical screening prior to inclusion in the study and standard chemistry and hematology tests were performed at clinical chemistry lab of the Leiden University Medical Center. BMI was restricted between 18 and 28 inclusive. Subjects for the YH, EH and YS groups were excluded if the following risk factors for CAD were present: high cholesterol, smoking, diabetes, hypertension or familial risk. Subjects for the YH and EH groups were considered healthy when no abnormalities were found in the urinalysis, hematology and chemistry lab tests, including liver enzymes, kidney function markers, cardiovascular markers (cholesterol, triglycerides, lipoprotein A, NTproBNP), CRP and coagulation markers. Furthermore, no abnormalities in the physical examination, including blood pressure and electrocardiogram and in the medical history were found.

PBMC ISOLATION AND CRYOPRESERVATION

PBMCs were isolated using Cell Preparation Tubes (Becton Dickinson, Frankin Lakes, NJ, USA), according to manufacturer's protocol. In short, tubes are centrifuged at 1800xg for 30 minutes after which the PBMCs were removed. PBMCs were washed twice using PBS (pH 7.2, Gibco, Thermo Fisher Scientific, Waltham, MA, USA). PBMCs were dissolved in heat inactivated fetal bovine serum (Gibco, Thermo Fisher Scientific), containing 10% DMSO (Sigma-Aldrich, Deisenhofen, Germany). PBMCs were frozen using a CoolCell at -80°C, prior to storage in liquid nitrogen.

B CELL ISOLATION AND STIMULATION

B cells were isolated directly from whole blood with the Easysep Direct B cell isolation kit using the RoboSep (Stemcell, Vancouver, Canada). Purity was assessed by flow cytometry using CD45-PE (clone: HI30) and CD19-APC (clone: HIB19) antibodies, and was >90% for all samples. B cells were stimulated with CpG class B (ODN2006, Invivogen, Toulouse, France) or PBS for 24 hours. After 24 hours expression of activation markers CD69, CD80 and CD86 were measured by flow cytometry, using CD69-AF700 (clone: FN50), CD80-PE (clone: 2D10) and CD86-APC (clone: BU63). All antibodies were from Biolegend (San Diego, CA, USA).

WHOLE BLOOD STIMULATION AND CYTOKINE MEASUREMENT

Heparinized whole blood was stimulated with 2ng/ml lipopolysaccharide (LPS O111:B4, Sigma-Aldrich) for 6 hours or PMA and ionomycin (both Sigma-Aldrich) + Brefeldin A (Thermo Fisher) for 4 hours at 37°C and 5% CO_2. PMA and ionomycin stimulated samples were used for intracellular cytokine staining. LPS stimulated samples were centrifuged and supernatant was collected. Cytokines in supernatant were measured using the LegendPlex virus response panel (Biolegend) according to manufacturer's manual.

FLOW CYTOMETRY

Red blood cell lysis was performed on whole blood samples using RBC lysis buffer (Thermo Fisher Scientific). Leukocytes were stained with fluorochrome labeled antibodies as indicated in supplementary table I. Intracellular staining was performed after fixation and permeabilization with IC fixation buffer and permeabilization buffer (both Thermo Fisher Scientific). Samples were measured on a MACSQuant 16 analyzer (Miltenyi Biotec, Bergisch-Gladbach, Germany) and analyzed using Flowlogic software (Inivai, Mentone, Australia).

IMMUNOGLOBULIN ELISAS

K_2EDTA plasma antibody levels to PC-BSA and $CUSO_4$-oxidized LDL (OXLDL) were measured by chemiluminescent ELISA as reported previously.[11] In brief, PC-BSA (Biosearch Technologies) and OXLDL were coated at 5µg/ml in PBS/EDTA. IgM antibodies were measured at a dilution of 1:500 and IgG antibodies at 1:1000.

OXLDL IGM B CELL ELISPOT

IgM B cell ELISPOTS were performed on thawed PBMCs, using the IgM ELISPOT Basic kit (Mabtech, Stockholm, Sweden). PBMCs were stimulated with R848 and IL-2 for 3 days to stimulate antibody production. ELISPOT plates (Multiscreen IP filter plate, PVDF membrane, Sigma-Aldrich) were coated with 15µg/ml OXLDL (Thermo Fisher Scientific) or anti-IgM antibody. PBMCs were incubated in the coated ELISPOT plates at 200.000 (OXLDL) or 12.500 cells/well (anti-IgM) for 24 hours in X-Vivo15 medium (Lonza, Ambiose, France) with 1% penicillin and streptomycin (Thermo Fisher Scientific). Plates were developed using detection antibodies from the kit and developed using NCIB/NBT substrate (Mabtech). Spots were counted using the Bioreader 6000-E β (Biosys, Karben, Germany). Samples were tested in triplicate. Mean OXLDL spots/total IgM spot count were calculated.

PROTEOMICS

92 proteins were measured in multiplex in K_2EDTA plasma by Olink (Uppsala, Sweden) using the pre-designed immune response panel. Protein levels were measured using oligonucleotide labeled antibodies. When 2 antibodies were in proximity, the DNA sequence was enhanced by real time PCR. The results were expressed on a log2 scale as normalized protein expression (NPX).

STATISTICAL ANALYSIS

The means of all groups were compared to each other, and pairwise differences were calculated using one-way ANOVA (with Dunnett's multiple testing adjustment) or Kruskal-Wallis test (Dunn's multiple testing adjustment) when normality assumption failed. P values ≤0.05 obtained from above tests were considered statistically significant. Data are expressed as arithmetic mean ± standard deviation. Analysis and visualization were done using Graphpad Prism version 9.2.0 (Graphpad Sofware, San Diego, CA, USA).

Proteomics data were analyzed using one-way ANOVA (with Tukey's multiple testing adjustment for multiple groups) and corrected for multiple testing using Benjamini-Hochberg procedure using SPSS (IBM, Armonk, NY, USA). Hierarchical cluster analysis was performed using agglomeration method of Ward's minimum variance on Euclidian dissimilarities matrix based on data in original scale. Hierarchical cluster analysis was conducted using functions {R package} in Rstudio (Boston, MA, USA) for clustering and visualization of its results in forms of a heatmap are hclust {stat} and pheatmap {pheatmap} respectively.

Results

In total, 108 subjects were included in the study as shown in Figure 1 and Table I (subject demographics). The main exclusion factor for elderly healthy volunteers was an increased cholesterol level (reference range <5.00 mmol cholesterol/L and <3.00 mmol LDL cholesterol/L) or hypertension (systolic >140 mmHG and/or diastolic >90 mmHG in supine position after 5 minutes rest). Twelve of the 27 coronary artery disease patients were smokers. 26 of 27 were taking medication related to CVD, 24/27 used NSAIDS, 21/27 used statins, 8/27 used beta blockers, 4/27 used calcium blockers, 4/27 used angiotensin II blockers and 2/27 used diuretics.

ELEVATED TOTAL LEUKOCYTE AND NEUTROPHIL NUMBERS IN AGED VOLUNTEERS, ADDITIONALLY ENHANCED BY SMOKING

First, we compared the absolute numbers of circulating leukocytes and its subsets between the groups. In heavy smokers, the total number of leukocytes was significantly higher compared to all other groups (8.3×10^9/L ± 1.6 for HS, vs $5.4 \pm 1.3 \times 10^9$/L for YH, $6.4 \pm 1.4 \times 10^9$/L for EH, $6.1 \pm 1.6 \times 10^9$/L for YS, $6.1 \pm 1.5 \times 10^9$/L for CAD) (Figure 2A). The number of lymphocytes was lower in the CAD group compared to young healthy volunteers and heavy smokers ($1.6 \pm 0.3 \times 10^9$/L for CAD, vs $1.89 \pm 0.47 \times 10^9$/L for YH and $2.1 \pm 0.5 \times 10^9$/L for HS), while the numbers were significantly lower in the elderly healthy group ($1.6 \pm 0.5 \times 10^9$/L) compared to the heavy smokers (Figure 2B). The number of neutrophils was significantly higher in elderly healthy subjects compared to young healthy subjects ($4.1 \pm 1.3 \times 10^9$/L for EH, vs $3.0 \pm 1.1 \times 10^9$/L for YH) (Figure 2C), and even higher in the heavy smoker group ($5.3 \pm 1.6 \times 10^9$/L). Furthermore, neutrophil numbers were significantly higher in the heavy smokers compared to the CAD group ($3.8 \pm 1.4 \times 10^9$/L). No differences were observed in numbers of circulating monocytes (Figure 2D).

LOWER LEVELS OF CD16$^+$ MONOCYTES AND PLASMACYTOID DENDRITIC CELLS IN SMOKING VOLUNTEERS

Upon flow cytometry analysis of the myeloid cells, a lower percentage of non-classical (CD14-CD16$^+$) monocytes was seen in the heavy smoker and young smoker groups, compared to young healthy volunteers (0.9 ± 0.5% for HS, 1.5 ± 0.9% for YS compared to 2.7 ± 2.0% for YH). Classical (CD14$^+$CD16$^-$) and intermediate (CD14$^+$CD16$^+$) monocytes did not differ between the groups (Figure S1A). The percentage of plasmacytoid DCS (HLA-DR$^+$ CD14$^-$ CD123$^+$) was significantly lower in heavy smokers compared to elderly healthy volunteers and young smokers (0.15 ± 0.08% for HS vs. 0.36 ± 0.16% for YS and 0.33 ± 0.19% for YH, Figure 2D).

To investigate the functionality of (mainly) myeloid cells, whole blood was stimulated with TLR4 ligand LPS, after which cytokine release was measured in the supernatant. A significant decrease in TNFα release was found in elderly healthy volunteers and patients with CAD compared to young healthy (Figure 3B, see Table S2 for mean and SD). GM-CSF was significantly decreased in the young smokers and CAD patient groups compared to young healthy volunteers. The interleukins IL-8, IL-6 and IL-10 were significantly decreased in the CAD group compared to young healthy volunteers. No differences were observed in IL-1β release (Figure S1B).

REDUCED NAÏVE T CELL LEVELS IN ELDERLY HEALTHY VOLUNTEERS, WHILE SMOKERS HAVE AN ELEVATED RELATIVE PRO-INFLAMMATORY TH1 FRACTION

Circulating levels of T cell subsets were assessed using flow cytometry. CD3$^+$ T cells as percentage of CD45 were lower in aged volunteers (25.3 ± 12.1% for EH, vs 38.1 ± 15.1% for YH) (Figure 4A). This difference can be attributed to a strong decrease in CD8$^+$ cytotoxic T cells (4.1 ± 2.8% for EH, vs 11 ± 5.6% for YH), which was also observed in the heavy smoker and CAD patient groups (6.1 ± 4.1% for HS, 5.1 ± 4.1% for CAD). Furthermore, the CD4/CD8 ratio, calculated using percentages of CD3$^+$ cells, was increased in the elderly healthy and CAD patient groups (5.7 ± 4.1 for EH, 4.6 ± 2.4 for CAD vs. 2.7 ± 1.0 for YH, 2.5 ± 1.0 for YS and 3.3 ± 1.8 for CAD). As expected, the relative number of naive cells (CCR7$^+$ CD45RO$^-$) in both the CD4$^+$ and CD8$^+$ T cell populations decreases significantly with age, while the percentage of memory cells increases (Figure 4B$^+$C). Interestingly, both CD4$^+$ and CD8$^+$ central memory (CCR7$^+$ CD45RO$^+$) cell levels were elevated in heavy smokers compared to young volunteers (22.3 ± 8.9% for CAD vs. 12.1 ± 6.8% for YH and 10.8 ± 5.8% for YS, CD8: 8.6 ± 7.9% for CAD vs. 3.7 ± 3.4% for YH and 3.3 ± 2.7% for YS) (Figure 4B$^+$C).

Upon focusing on the TH subsets, we observed that heavy smokers had a larger TH1 (CXCR3$^+$ CCR6$^-$) fraction (45.4 ± 7.1% for HS vs 30.8 ± 12.8% for YH), and a smaller TH2 (CXCR3$^-$ CCR6$^-$) fraction of CD4$^+$ T cells (32.7 ± 5.9% for HS vs 45.4 ± 11.4% for YH) (Figure 5A). No differences were observed in the regulatory T cell subset (Figure S2).

To evaluate the functionality of CD4$^+$ T cells in the different populations, whole blood was stimulated with PMA and ionomycin for 4 hours in the presence of brefeldin A for intracellular cytokine staining in T cells (Figure 5B). Corresponding to the elevated TH1 cells, more IFNγ producing CD4$^+$ cells were observed in heavy smokers compared to all other groups (9.7 ± 9.1% for HS vs. 3.7 ± 3.8% for YH, 2.7 ± 3.4% for EH, 3.4 ± 4.0% for YS and 2.9 ± 4.1% for CAD). Although TH17 (CXCR3$^-$ CCR6$^+$) cell percentages were unaltered in heavy smokers (Figure 5A), we observed an elevated percentage of IL-17 producing CD4$^+$ T cells in this group, compared to young healthy volunteers (10.2 ± 9.2% for HS vs. 5.7 ± 3.0% for YH).

Accumulation of CD57$^+$ and CD28^{null} T cells in the blood has been described upon ageing.[12,13] Indeed, we found increased CD57$^+$ cells within the CD8$^+$ T cells of elderly healthy and patients with CAD, compared to young healthy volunteers (29.3 ± 15.5% for EH; 27.5 ± 13.9% for CADvs. 16.0 ± 13.8% for YH) (Figure 5C). Similarly, we observed a trend (p=0.06) towards increased CD28^{null} cells in the CD8$^+$ T cell population in patients with CAD (28.0 ± 19.7%) compared to young healthy volunteers (18.5.0 ± 15.6%). Although we did not observe any significant differences in CD57$^+$ cells within the CD4$^+$ T cell compartment, we found elevated levels of CD28^{null} cells in CD4$^+$ T cells of elderly healthy compared to young healthy volunteers (5.3 ± 6.7% for EH vs. 1.3 ± 1.4% for YH). The percentage of CD57$^+$ and CD28^{null} CD4$^+$ T cells in patients with CAD remained unaltered.

PATIENTS WITH CAD HAVE LOWER LEVELS OF CIRCULATING IGM

Next, we investigated humoral immunity, starting with circulating B cell subsets. No differences were observed in naive, CD19$^+$ CD27$^+$ CD43$^+$ B1 cells, non-class-switched or class-switched B cells for any group compared to the healthy young volunteers (Figure S3). The percentage of transitional B cells was significantly lower in aged volunteers (4.0 ± 2.2% for EH), young

smokers (3.9 ± 2.2% for YS) and heavy smokers (3.2 ± 1.7% for HS) compared to young healthy volunteers (6.9 ± 4.8%). No significant differences were found for $CD5^{+}$ $CD1d^{hi}$ regulatory B cells (Figure 6A).

Next, we measured circulating immunoglobulin levels. We observed a significantly lower concentration of total serum IgM in the CAD patient group (0.63 ± 0.24 g/L) compared to young healthy volunteers (1.07 ± 0.40 g/L), young smokers (1.01 ± 0.43 g/L) and heavy smokers (1.07 ± 0.53 g/L) (Figure 6B). No differences were found for total IgG serum concentrations (Figure 6B). We also measured OXLDL-specific immunoglobulins, as these immunoglobulins can play both anti- (IgM) as well as pro-atherogenic (IgG) roles. The level of OXLDL-specific IgM was significantly lower in the CAD patient group compared to both young healthy volunteers and young smokers (6146 ± 4336 RLU for CAD vs. 13828 ± 7620 RLU for YH and 12636 ± 8349 RLU for YS), while no differences were observed in OXLDL-specific IgG values (Figure 6C). Since phosphorylcholine (PC) is a specific epitope of OXLDL, PC-specific antibodies were measured as well. In elderly healthy volunteers, a lower level of PC-specific IgM was found (8176 ± 5114 RLU for EH vs. 13281 ± 5512 RLU for YH), while the PC-specific IgG signal was elevated (34925 ± 7138 RLU for EH vs. 29502 ± 6281 RLU for YH) (Figure 6D). The lowered PC-IgM was also observed in patients with CAD (7696 ± 4908 RLU).

To assess the number of OXLDL-IgM secreting B cells present in PBMCs, PBMCs were stimulated with R848 (TLR7/8 agonist) and IL-2 for 2 days to activate memory B cells, after which the number of IgM and OXLDL-specific IgM secreting B cells was measured using ELISPOT. The percentage of OXLDL-specific IgM secreting B cells of total IgM secreting B cells is shown in Figure 6E. Although the circulating levels of OXLDL-IgM are decreased in the patients with CAD, the number of OXLDL-specific IgM secreting B cells is not.

To assess the activation capacity of B cells, B cells were stimulated with CpG class B for 24 hours, and activation was measured by CD69, CD80 and CD86 expression using flow cytometry. No differences were observed between groups for the expression of CD69 and CD86 (Figure S3). Expression of the co-stimulation marker CD80 was significantly increased on B cells in the CAD patient group, compared to young healthy volunteers and young smokers (18.59 ± 6.15% for CAD vs. 12.16 ± 8.88% for YH and 11.59 ± 6.91% for YS) (Figure 6F).

ELEVATED TREM1 AND CCL11 PLASMA LEVELS IN ELDERLY (SMOKING) SUBJECTS AND PATIENTS WITH CAD

Plasma samples were analyzed for inflammatory proteins using the Olink immune response panel (Figure 7). A significantly elevated level of triggering receptor expressed on myeloid cells 1 (TREM1), C-C motif chemokine ligand 11 (CCL11) and leukocyte immunoglobulin like receptor B4 (LILRB4) was seen in plasma of elderly healthy volunteers compared to young healthy volunteers. In heavy smokers the elevation of TREM1 and CCL11 was more profound, and also significantly elevated levels of IL-6 and lysosomal associated membrane protein 3 (LAMP3) were found. Patients with CAD show a similar profile to elderly healthy volunteers. When clustering all data using Ward's hierarchical clustering, two clusters were found, consisting of samples from all subject groups (Figure 7B). This shows that the protein profiles do not clearly differentiate the groups from each other.

Discussion

Immunomodulation has shown to be a valuable therapeutic approach in atherosclerosis in animal studies.[14] Recently, clinical trials with immunomodulatory drugs have been performed in cardiovascular patient populations. In the CANTOS and LODOCO trials[8,9] a significant reduction in cardiovascular risk was observed, while low dose methotrexate (CIRT) did not reveal a therapeutic effect.[10] Since using recurrent cardiovascular events as endpoint requires large patient groups, clinical evaluation of immunomodulatory investigational drugs for atherosclerosis could benefit from relatively small phase 1B studies, in well-characterized populations carrying cardiovascular risk factors (ageing, smoking). We conducted an observational study evaluating the effects of age, smoking and cardiovascular disease on a broad set of immune endpoints. We aimed to identify immune markers differentiating populations with cardiovascular risk factors from young healthy volunteers, thereby identifying the most optimal population to be used as 'disease model' for future phase 1B studies with immunomodulatory drugs.

First, we assessed the effects of ageing on circulating immune cell populations. A hallmark of immunosenescence is decreased lymphopoiesis

and accumulation of senescent cells.[15,16] Indeed, we observed significantly reduced total lymphocyte numbers, total CD3$^+$ T cells and CD8$^+$ T cells in aged populations. Furthermore, we observed an increase in senescent CD4$^+$CD28^{null} cells in elderly healthy volunteers and elevated CD8$^+$CD57$^+$ T cells in both elderly healthy volunteers and stable CAD patients, compared to young individuals. Previous studies have shown that a high frequency of senescent CD28^{null} and CD57$^+$ T cells in peripheral blood and/or atherosclerotic plaques strongly associate with hyperglycemia, acute cardiovascular events and mortality.[17-19] Interestingly, statin treatment has been shown to reduce elevated senescent CD4$^+$CD28^{null} cells,[17] which might attribute to unaltered CD4$^+$CD28^{null} levels in statin treated CAD patients compared to young healthy, whereas levels in elderly healthy increased. Whether statin treatment might have reduced the frequency of CD8$^+$ CD57$^+$ T cells in the CAD patient group, remains to be elucidated.

Besides ageing, smoking is an important risk factor for atherosclerosis development as well.[20]An increase in central memory T cells was observed in the heavy smoker group, which has been described previously, where the amount of CD45RO$^+$ T cells was strongly correlated to the amount of pack years.[21] In addition, an increase in the TH1 fraction was seen in this population, with a corresponding increase in IFNγ producing T cells.

The elderly healthy group resembled the CAD group quite well, with the exception of B cell effects, and myeloid cell function after LPS stimulation. A lower level of OXLDL-specific IgM was found in patients with CAD compared to young healthy volunteers, as was also shown previously.[22,23] OXLDL IgM plays an atheroprotective role, it facilitates the clearance of apoptotic cells,[7,24] while preventing foam cell formation by blocking OXLDL uptake by macrophages,[25] and possesses antithrombotic properties.[26] Furthermore, OXLDL IgM has an inverse relation to human intima media carotid artery thickness.[27] A reduced level of PC-specific IgM was also found in the patients with CAD and elderly healthy volunteers, consistent with a decline of these type of IgM with age, which are considered atheroprotective.[28,29]

Myeloid cell functionality was evaluated by performance of whole blood LPS challenges. Overall, cytokine release was significantly reduced in the CAD group compared to young healthy volunteers. This decrease in cytokine production in the stable CAD group could be attributed to medication use. Statins are known to have an immunosuppressive effect[30], supporting its use in graft versus host disease,[31] multiple sclerosis,[32] and heart transplant patients.[33] However, it has also been shown that statins do not affect cytokine production by monocytes after LPS stimulation.[34]

Finally, we compared the study groups for circulating inflammatory markers by proteomics. A significant increase in plasma TREM1 and CCL11 was found in elderly volunteers compared to the young healthy group. TREM1 is an enhancer of pro-inflammatory innate immune responses and plays an important role during infection. Increased TREM1 levels have been found in Alzheimer's disease,[35] Parkinson's disease[36] and sepsis,[37] but to our knowledge this is the first time an increase in plasma TREM1 levels is found in ageing. CCL11 (eotaxin) plasma levels have been found to positively correlate to the score of coronary artery stenosis.[38] In both heavy smokers and patients with CAD the increase in TREM1 and CCL11 was also found, indicating that an increase in these proteins may be potential risk factors for atherosclerosis development. Both groups had increased IL-6 plasma levels as well, IL-6 being one of the hallmarks of inflammaging and influenced by smoking.[39,40] Increased plasma IL-6 levels correlates with risk for CAD,[41-43] furthermore treatment of patients with high atherosclerotic risk with ziltivekimab, a human monoclonal antibody against IL-6, resulted in a reduction of inflammation biomarkers relevant to atherosclerosis.[44] LAMP3 clearly distinguished the heavy smoker group from the other groups. LAMP3 is mainly expressed in mature dendritic cells, mostly in the lysosomes. Little is known about the role of LAMP3 in disease, although an association between LAMP3 expression andserum auto-antibodies in Sjögren's syndrome was found.[45]

There are several limitations to this study. TLR4, through which LPS primarily signals, is not solely expressed on myeloid cells,[46] so we cannot exclude the possibility that non-myeloid cells may have contributed to the observed cytokine responses. Furthermore, differences in whole blood composition between the groups could attribute to the observed differences in cytokine production between the groups. Another limitation of this study is the small sample size, and the relatively high analytical variation of several endpoints. While clear immunological differences were found between study groups, smaller differences could have remained unnoticed. However, it was the specific aim of this study to identify differentiating

immune endpoints at sample sizes resembling future phase 1B studies, which are commonly conducted at relatively low volunteer numbers. To summarize the study outcomes, we have visualized the main impact of aging, smoking and CAD on myeloid, T and B cell subsets/functionality, and on circulating immune markers (Figure 8). To conclude, significant effects of ageing and smoking on the immune system could be identified at relatively low group sizes of 11-30 subjects per group.Based on our data, both the aged and heavy smoking but otherwise healthy population, could be valuable for evaluation of the effects of future immunomodulatory drugs targeting atherosclerosis instead of patients with CAD, where the elderly healthy group corresponds most with the CAD patient group.

Table 1 Demographics

Group	Young Healthy (n=30)	Elderly Healthy (n=20)	Young Smoker (n=20)	Heavy Smoker (n=11)	Coronary Artery Disease patients (n=27)	P value
age, years, median (Q25–Q75)	22 (19–22.3)	70.5[a,b,c] (67–77.5)	22 (21–23.8)	55[a,b] (48–68)	69.5[a,b,c] (66–73.3)	<0.0001
BMI, kg/m^2, median (Q25–Q75)	22.5 (20.6–23.3)	24.6 [a,b] (22.8–25.4)	21.7 (20.7–23.8)	25.2 [a,b] (22.8–27.2)	24.8 [a,b] (23.5–26.4)	<0.0001
diastolic BP (supine) mmHG, mean, ± SD	69.9 ± 4.8	81.4 ± 8.9 [a,b]	72.4 ± 6.7	83.1 ± 5.5 [a,b]	80.7 ± 9.3 [a,b]	<0.0001
systolic BP (supine) mmHG, mean, ± SD	118 ± 9.2	133.4 ± 15[a]	122 ± 10.3	127.6 ± 11.4	131.8 ± 17.6[a]	0.0002
cholesterol, mmol/L, mean, ± SD	3.84 ± 0.54	4.53 ± 0.43[a,b]	3.88 ± 0.57	5.19 ± 0.97[a,b]	4.22 ± 0.98[c]	<0.0001
LDL, mmol/L, mean, ± SD	2.19 ± 0.36	2.60 ± 0.37[b]	2.10 ± 0.37	3.17 ± 0.95[a,b]	2.30 ± 0.93[c]	0.0002
HDL, mmol/L, mean, ± SD	1.29 ± 0.21	1.46 ± 0.28	1.40 ± 0.32	1.32 ± 0.28	1.50 ± 0.33	0.1249
triglycerides, mmol/L, mean, ± SD	0.78 ± 0.32	1.04 ± 0.26[c]	0.83 ± 0.30	1.55 ± 1.06[a,b]	1.03 ± 0.50[c]	0.0003
Apolipoprotein A, g/L, mean, ± SD	1.32 ± 0.17	1.44 ± 0.21	1.33 ± 0.21	1.37 ± 0.22	1.49 ± 0.25[a]	0.03
NTproBNP, mean, ± SD	26.52 ± 17.16	94.38 ± 87.96[a,b]	29.40 ± 22.59	48.96 ± 43.53	175.5 ± 182.6[a,b,c]	<0.0001

a=significantly different to YH. b=significantly different to YS, c= significantly different to HS

Figure 1 CONSORT flowchart.

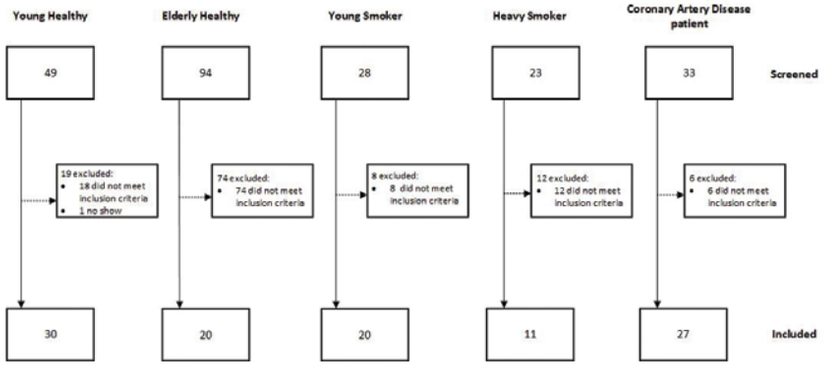


Figure 2 Effect of ageing and smoking on absolute circulating leukocytes. Amount of leukocytes (A), lymphocytes (B), neutrophils (C), monocytes (D) in circulation as measured by sysmex. LLON=lower limit of normal, ULON=upper limit of normal. Mean ± SD are shown. LLON=lower limit of normal. ULON=upper limit of normal. YH=young healthy, EH=elderly healthy, YS=young smoker, HS=heavy smoker, CAD=coronary artery disease patient. Open circles in the CAD group represent smokers. Statistics was performed using one-way ANOVA with Dunnett's post hoc test, means were compared to the YH group. P values ≤0.05 are considered significant. * P≤0.05, ** P≤0.005, *** P≤0.001, **** P≤ 0.0001.

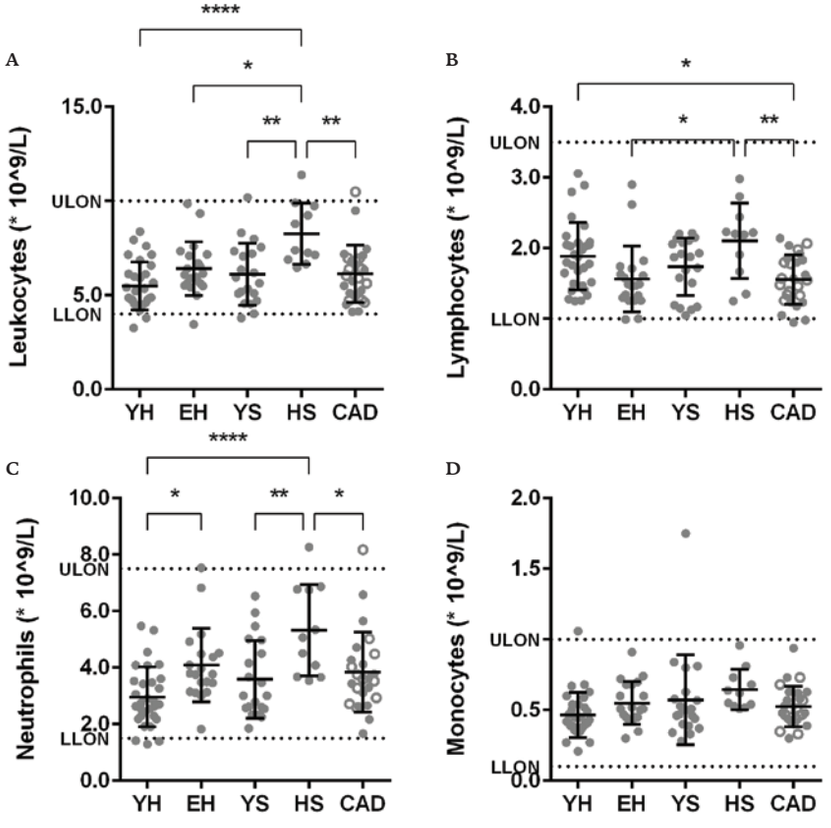


Figure 3 Effect of ageing and smoking on myeloid cells. Percentage of non-classical monocytes (CD14$^-$ CD16$^+$) and plasmacytoid dendritic cells (HLA-DR$^+$, CD19-, CD14-, CD123$^+$) in whole blood as assessed by flow cytometry (A). Whole blood was stimulated with 2 ng/ml LPS and cytokine release was measured using LegendPlex (B). YH=young healthy, EH=elderly healthy, YS=young smoker, HS=heavy smoker, CAD=coronary artery disease patient. Open circles in the CAD group represent smokers. Statistics was performed using one-way ANOVA with Dunnett's post hoc test, means were compared to the YH group. P values ≤0.05 are considered significant. * P≤0.05, ** P≤0.005, *** P≤0.001, **** P≤ 0.0001.

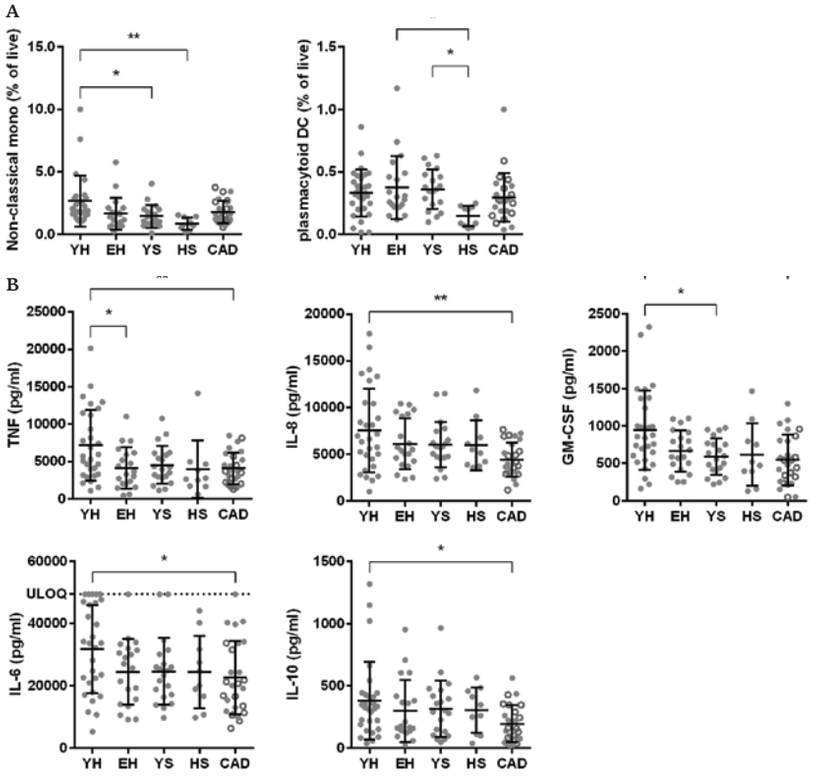


Figure 4 Effect of ageing and smoking on T cells and memory subsets. Percentage of CD3$^+$, CD4$^+$ and CD8$^+$ T cells of total CD45$^+$ leukocytes was measured by flow cytometry (A). Naïve (CCR7$^+$ CD45RO$^-$) and memory subsets (central memory: CCR7$^+$ CD45RO$^+$, effector memory: CCR7$^-$ CD45RO$^+$) were quantified for CD4$^+$ T cells (B) as well as CD8$^+$ T cells (C). YH=young healthy, EH=elderly healthy, YS=young smoker, HS=heavy smoker, CAD=coronary artery disease patient. Open circles in the CAD group represent smokers. Statistics was performed using one-way ANOVA with Dunnett's post hoc test, means were compared to the YH group. P values ≤0.05 are considered significant. * P≤0.05, ** P≤0.005, *** P≤0.001, **** P≤ 0.0001.

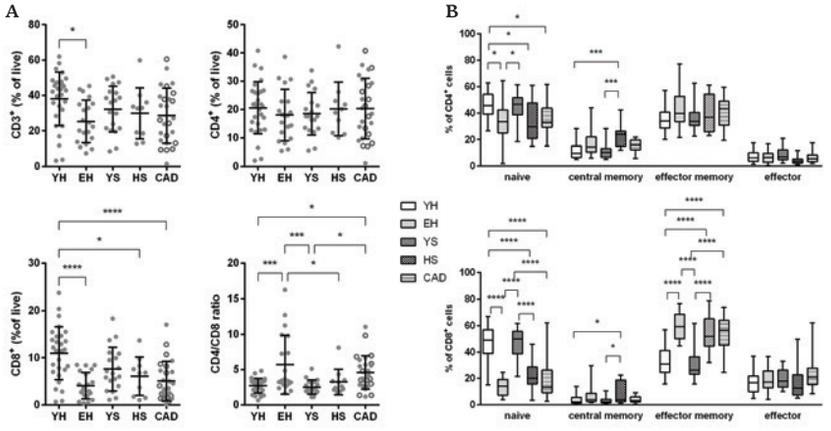


Figure 5 Effect of ageing and smoking on TH subsets and T cell senescence. Percentage of TH1 (CXCR3$^+$ CCR6$^-$), TH2 (CXCR3$^-$ CCR6$^-$) and TH17 (CXCR3$^-$ CCR6$^+$) cells were measured by flow cytometry (A). Cytokine production in CD4$^+$ T cells was measured after stimulation with PMA + ionomycin in the presence of BrefA, using flow cytometry (B). Both CD4$^+$ as well as CD8$^+$ T cells were assessed for senescence markers CD57 (positive) and CD28 (negative) (C). YH=young healthy, EH=elderly healthy, YS=young smoker, HS=heavy smoker, CAD=coronary artery disease patient. Open circles in the CAD group represent smokers. Statistics was performed using one-way ANOVA with Dunnett's post hoc test, means were compared to the YH group.P values ≤0.05 are considered significant. * P≤0.05, ** P≤0.005, *** P≤0.001, **** P≤ 0.0001.

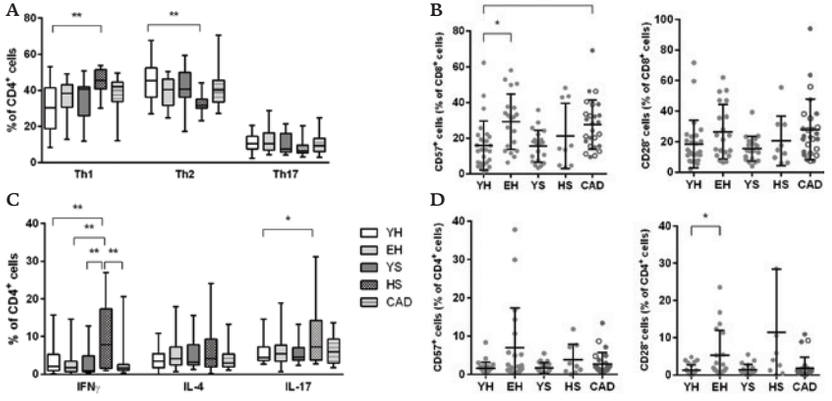


Figure 6 Effect of ageing and smoking on B cells. Number of CD24^{hi} CD38^{hi} and CD1d^{hi} CD5$^+$ regulatory B cells, evaluated by flow cytometry (A). Total IgM and IgG as assessed at the clinical chemistry lab at LUMC (B). OXLDL specific IgM and IgG as well as PC IgM and IgG as assessed by ELISA (C). OXLDL secreting B cells were measured using ELISPOT after 3 days of stimulation with R848 and IL-2 and are expressed as percentage of total IgM secreting B cells (D). CD80 expression was measured by flow cytometry on isolated B cells after 24 hours of stimulation with CpG B (E). YH=young healthy, EH=elderly healthy, YS=young smoker, HS=heavy smoker, CAD=coronary artery disease patient. Open circles in the CAD group represent the smokers. Statistics was performed using one-way ANOVA with Dunnett's post hoc test or Kruskall-Wallis test with Dunn's post hoc test, means were compared to the YH group. P values ≤0.05 are considered significant. * P≤0.05, ** P≤0.005, *** P≤0.001, **** P≤ 0.0001.

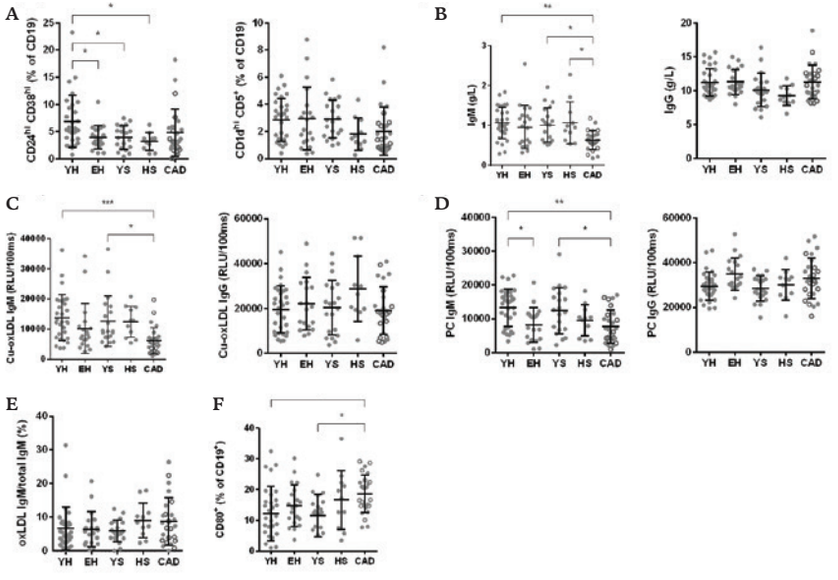
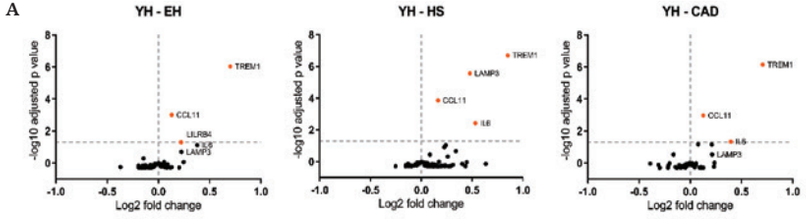


Figure 7 Effect of ageing and smoking on levels of circulating inflammatory proteins. Volcano plots of proteomics (A), EH vs YH, HS vs YH and CAD vs YH. Horizontal line depicts significance, data points depicted in orange show increased serum expression. Heatmap of agglomerative hierarchical clustering using Ward's method of proteomics data (B). YH (n=19), EH (n=19), YS (n=19), HS (n=11), CAD (n=20). YH=young healthy, EH=elderly healthy, YS=young smoker, HS=heavy smoker, CAD=coronary artery disease patient.

A



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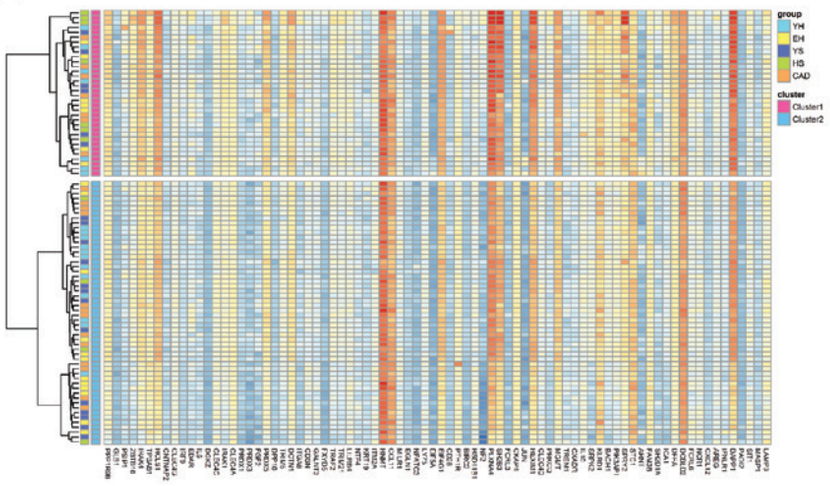

All mentioned supplementary figures and tables in this chapter can be found on the corresponding website by scanning this QR code.

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CHAPTER 3

In vitro and *in vivo* lipopolysaccharide-driven activation of human neutrophils in healthy volunteers

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Graphical abstract

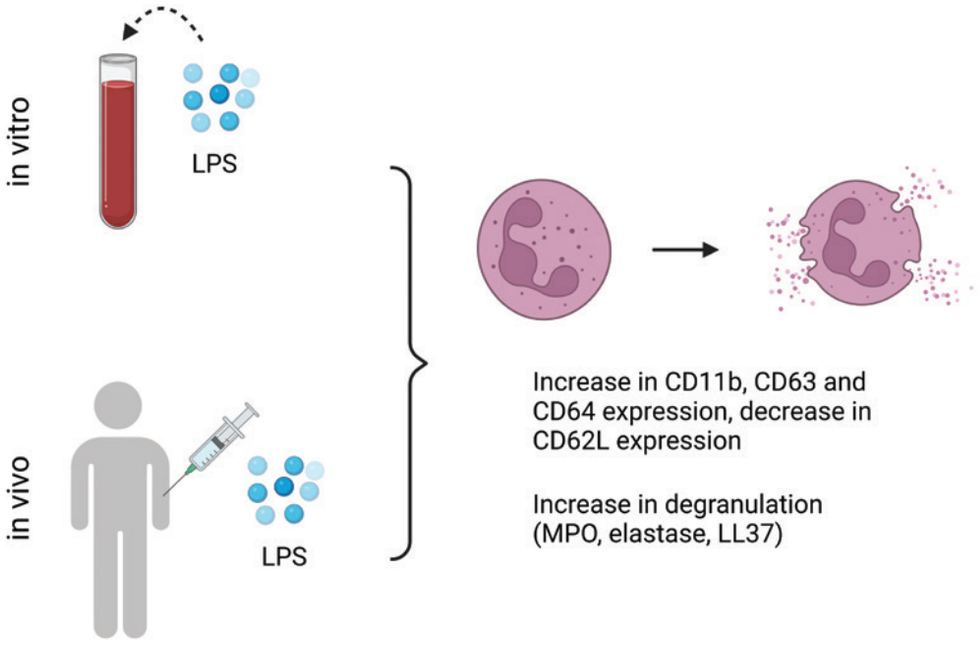


Figure is created using biorender.com.

Abstract

Neutrophils are an emerging target for therapeutical intervention in both autoimmune diseases as well as cancer. However, evaluating investigational compounds in healthy humans remains challenging, since this population lacks constitutive neutrophil activation. Induction of neutrophil activation via intravenous administration of lipopolysaccharides (LPS) can be a potent strategy to overcome this challenge. Furthermore, LPS stimulation can be performed *ex vivo* during clinical trials, and *in vitro* for pre-clinical analysis. Therefore, we aimed to provide a time course of the neutrophil response after *in vivo* LPS administration using samples from human endotoxemia clinical studies and compared this to *in vitro* LPS stimulated whole blood cultures. We performed shotgun proteomics on *in vivo* stimulated neutrophils, and measured neutrophil activation by flow cytometry using CD11b, CD62L, CD63 and CD64 as activation markers and elastase, MPO and LL37 levels as degranulation markers. The numbers of neutrophils rapidly increased after LPS administration, while monocyte and lymphocyte numbers significantly decreased. In line, we found significant increases in neutrophil activation and degranulation markers both *in vitro* as well as *in vivo*, which all returned to baseline within 24 hours. Degranulation proteins rapidly increase after LPS administration (1 hour after exposure) *in vivo*, while higher concentrations of LPS were necessary *in vitro*. Lastly, shotgun proteomics revealed little but significant differences in the neutrophil proteome after *in vivo* LPS administration, pointing to degranulation after LPS stimulation. In general, neutrophils show similar activation *in vitro* and *in vivo*. Both, the *in vitro* whole blood LPS stimulation assay and the human endotoxemia model, could be valuable tools for evaluation of the effects of future drugs modulating neutrophil responses during preclinical and clinical development.

Introduction

Neutrophils are the first responders upon infection. They are professional phagocytes, with short life-spans of multiple days.[1] Neutrophils carry granules, which can be released upon activation and contain proteolytic enzymes including neutrophil elastase and myeloperoxidase (MPO), reactive oxygen species, and anti-microbial peptides such as LL37. To prevent tissue damage by excessive neutrophil activity, apoptosis of neutrophils is tightly regulated.[2] Besides their role in acute responses against pathogens, neutrophil activation has been found to contribute to several auto-immune diseases, amongst others rheumatoid arthritis[3] and systemic lupus erythematosus,[4] by inducing tissue damage via production of cytokines and chemokines and via the formation of NETs.[5] In atherosclerosis, neutrophils are involved in the onset of disease, mainly by accumulating in the vessel wall and attracting monocytes to the lesion site.[6] They also play a role in destabilization of the atherosclerotic plaque and atherothrombosis, as is illustrated bythe presence of neutrophils in unstable plaques.[7-9] Besides their role in auto-immune diseases, neutrophils have recently been implicated to play a role in cancer development and progression.[10] They can have both protumoural and antitumoural effects, indicating that a balance in neutrophil activation is required for homeostasis.[10] Because of their role in autoimmune diseases and in cancer progression, neutrophils have emerged as potential therapeutic target.[11] Inhibition of neutrophil activity can be accomplished at several levels, for example by blocking neutrophil migration or activation and thereby interfering with neutrophil accumulation at the site of inflammation. Furthermore, interference of neutrophil-specific cell death via neutrophil extracellular traps (NETosis), and blocking of neutrophil-specific proteins can be used to inhibit neutrophil activity.[11] Indeed, several drugs are in clinical development, such as a CXCR2 antagonist, a neutrophil elastase inhibitor and NETosis inhibitors.[11]

For novel drug candidates targeting neutrophils, specific (pre)clinical tests are required. Evaluation of the pharmacological activity of neutrophil-targeted investigational compounds in healthy humans is however challenging since this population does not have constitutive neutrophil activation. Induction of neutrophil activation via intravenous administration of lipopolysaccharides (LPS) can be a potent strategy to be able to test drug candidates. LPS activates neutrophils *in vivo*, leads to increasing neutrophil numbers in the circulation[12,13] and shifts the neutrophil population towards CD16^{dim} (banded) neutrophils and CD62L^{low} (hypersegmented) neutrophils, besides the CD16^{high}CD62L^{high} neutrophils found in homeostasis.[14] CD62L (L-selectin) is an adhesion molecule that is quickly downregulated by activated neutrophils via ectodomain shedding.[15] CD11b (integrin alpha M), a constitutively expressed adhesion molecule on myeloid cells,[16,17] is another marker for neutrophil activation, known to be upregulated by for instance LPS exposure.[18,19] As such, the human endotoxemia model may be a useful tool to study the pharmacodynamic effects of novel drugs on neutrophils in an early stage of drug development. However, a clear overview of the effects of *in vivo* LPS exposure on neutrophils is currently lacking.

Additionally, LPS stimulation of whole blood *ex vivo* during clinical trials or of isolated neutrophils *in vitro* in pre-clinical analysis are important approaches to be explored to assess drug effects on neutrophils. To be able to compare *in vivo* LPS effects with *in vitro* LPS effects on neutrophils, we aimed to provide a time course of the neutrophil response after *in vivo* LPS administration and compared this to the effects of LPS stimulation on neutrophil activation status *in vitro*. We used samples from a clinical trial using the human endotoxemia model and performed *in vitro* whole blood stimulations using different concentrations of LPS. In the endotoxemia samples, we performed shotgun proteomics to identify changes in the neutrophil proteome. Furthermore, we assessed neutrophil activation by flow cytometry using CD11b, CD62L, CD63 and CD64 as activation markers and measured neutrophil degranulation by measuring myeloperoxidase (MPO), elastase and LL37 levels.

Materials and Methods

HUMAN SAMPLES

For the *in vivo* LPS challenge response evaluation, samples from 2 clinical trials were used. Both studies were conducted after obtaining written informed consent in accordance with Good Clinical Practice guidelines and the Declaration of Helsinki. In one study, healthy male volunteers received LPS intravenously at a dosage of 2 ng/kg, in a study registered under ToetsingOnline number NL65264.056.18 and under ISRCTN number 13923422.[20] In the other study, healthy male and female volunteers

received i.v. LPS at 1 ng/kg. For both studies purified lipopolysaccharide prepared from *Escherichia coli*, 113:H10:K negative (U.S. Standard Reference Endotoxin), manufactured by List Biological Laboratories (Campbell, CA, USA) was used. Subjects were hydrated with glucose/saline (2.5% glucose/0.45% sodium chloride) from 2 hours prior to LPS administration until 6 hours afterwards. For the *in vitro* LPS whole blood challenges, blood from healthy male and female donors was collected by venipuncture after obtaining written informed consent in accordance with Good Clinical Practice guidelines and the Declaration of Helsinki. Neutrophil activation was analyzed by flow cytometry in CTAD anti-coagulated blood. For elastase, MPO and LL37 measurements, sodium heparin plasma samples were used. For proteomic analysis, blood was collected into K_2EDTA tubes (Becton Dickinson). The demographics of the subjects are summarized in Table I.

WHOLE BLOOD STIMULATION

Whole blood was incubated with 2 ng/mL, 10 ng/mL or 100 µg/mL LPS (from *E.coli* O111:B4, Sigma-Aldrich, Deisenhofen, Germany) for 1 hour at 37°C 5% CO_2.

FLOW CYTOMETRY

Whole blood was incubated with RBC lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA) for 10 minutes to lyse red blood cells. Next, the cells were washed once with phosphate buffered saline (PBS, Gibco, Thermo Fisher Scientific) and stained with fluorochrome labeled antibodies for 30 minutes on ice (see supplemental table I for a list of antibodies used and Figure S1 for the gating strategy). After staining, the cells were washed and measured on a MACSQuant 16 analyzer (Miltenyi Biotec, Bergisch Gladbach, Germany). Flow cytometry data was analyzed using Flowlogic software (Inivai, Melbourne, Australia).

ELISAS

Elastase, myeloperoxidase (MPO) and LL37 were measured by ELISA according to the user's manuals. Elastase and LL37 ELISAS were obtained from Hycult Biotech (Uden, the Netherlands), MPO ELISA from Bio-Techne (Abingdon, UK). ELISAS were read on a Varioskan LUX reader and analyzed using the SkanIt software (both Thermo Fisher Scientific).

NEUTROPHIL ISOLATION

Neutrophils were isolated directly from whole blood by negative selection using the EasySep™ direct human neutrophil isolation kit (Stemcell, Vancouver, Canada) according to user's manual. After isolation, the neutrophils were pelleted and snap frozen in liquid nitrogen until analysis.

SHOTGUN PROTEOMICS

Samples were mixed with loading buffer and loaded on an 8 % pre-cast RunBlue gel (Expedeon), and run at 100 V for 5 min. Large gel spots with unseparated proteins were stained with InstantBlue (Expedeon) and excised in one gel slice and destained using 70% 50 mM NH_4HCO_3 and 30% acetonitrile. Reduction was performed using 10 mM dithiotreitol dissolved in 50 mM NH_4HCO_3 for 30 min at 55°C. Next, the samples were alkylated using 55 mM chloroacetamide in 50 mM NH_4HCO_3 for 30 min at room temperature. Subsequently, samples were washed for 10 min with 50 mM NH_4HCO_3 and for 15 min with 100% acetonitrile. Protein digestion was performed by addition of sequencing-grade modified trypsin (Promega; 25 µL of 10 ng/mL in 50 mM NH_4HCO_3) and overnight incubation at 37°C. Peptides were extracted using 5% formic acid in water followed by a second elution with 5% formic acid in 75% acetonitrile. Samples were dried in a SpeedVac centrifuge and dissolved in 20 µL 5% formic acid in water for analysis with LC-MS/MS.

The samples were analyzed on a nanoLC-MS/MS consisting of an Ultimate 3000 LC system (Thermo Fisher Scientific, USA) interfaced with a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific). Peptide mixtures were loaded onto a 5 mm × 300 µm i.d. C18 PEPMAP100 trapping column with water with 0.1% formic acid at 20 µL/min. After loading and washing for 3 min, peptides were eluted onto a 15 cm × 75 µm i.d. C18 PEPMAP100 nanocolumn (Thermo Fisher Scientific). A mobile phase gradient at a flow rate of 300 nL/min and with a total run time 120 min was used: 2% – 40% of solvent B in 87 min; 40% – 80% B in 1 min; 80% B during 1 min, and back to 2% B. Solvent A was 100:0 water/acetonitrile (v/v) with 0.1% formic acid, and solvent B was 0:100 water/acetonitrile (v/v) with 0.1% formic acid. In the nanospray source a stainless-steel emitter (Thermo Fisher Scientific) was used at a spray voltage of 2 kV with no sheath or auxiliary gas flow. The ion transfer tube temperature was 250 °C. Spectra were acquired in data-dependent mode with a survey scan at

m/z 300 – 1650 at a resolution of 70 000 followed by MS/MS fragmentation of the top 10 precursor ions at a resolution of 17 500. Singly charged ions were excluded from MS/MS experiments and fragmented precursor ions were dynamically excluded for 20 s.

PEAKS Studio version Xpro (Bioinformatics Solutions, Inc., Waterloo, Canada) software was used to search the MS data against the from UniProt human reference proteome (downloaded 13 July 2021). Search parameters: trypsin digestion with up to 2 missed cleavages; fixed modification carbamidomethylation of cysteine; variable modification oxidation of methionine; precursor mass tolerance of 20 ppm; fragment mass tolerance of 0.02 Da. The false discovery rate was set at 0.1% on the peptide level. Label free quantitation of the different groups was performed with the PEAKS Q module.

STATISTICAL ANALYSIS

For *in vitro* data, the means of all groups were compared to each other, and pairwise differences were calculated using one-way ANOVA (with Dunnett's multiple testing adjustment). P values ≤0.05 obtained from above test were considered statistically significant. Data are expressed as arithmetic mean ± standard deviation. Clinical data were tested using the 1-way repeated-measures analysis of variance (with Dunnett's multiple testing adjustment), where post-dose time points were compared with baseline). P values ≤0.05 obtained from above test were considered statistically significant. Data are expressed as arithmetic mean ± standard deviation. For proteomics data, volcano plots were made in which 3 hour post-dose samples were compared to the baseline samples. All analysis and visualization were done using Graphpad Prism version 9.2.0 (Graphpad Sofware, San Diego, CA, USA).

Results

IN VIVO LPS ADMINISTRATION INCREASES CIRCULATING LEUKOCYTE AND NEUTROPHIL NUMBERS, WHILE MONOCYTES AND LYMPHOCYTES DECREASE

Twelve subjects were dosed with 1 ng/kg LPS. Blood samples were taken 15 minutes before dosing, and at 0.5, 1.75, 3, 6, 9, 24 and 48 hours after LPS administration for assessment of leukocyte differentiation. After LPS administration, total circulating leukocyte numbers significantly increased to $10.48*10^9$/L compared to $5.10*10^9$/L at baseline (Figure 1A). This is mainly caused by a 3.6-fold increase in circulating neutrophils, which peaked at 8 hours after LPS dosing ($9.03*10^9$/L compared to $2.51*10^9$/L at baseline, Figure 1B). In contrast to neutrophils, monocytes almost completely disappeared 1.75h after LPS administration ($0.07*10^9$/L compared to $0.46*10^9$/L at baseline) which normalized again at 6h after LPS administration ($0.68*10^9$/L, Figure 1C). The number of circulating lymphocytes also significantly dropped upon LPS administration, with the lowest point reached at 3 hours ($0.56*10^9$/L, compared to $1.90*10^9$/L at baseline, Figure 1D). All these effects on circulating leukocyte numbers normalized at 24 hours after LPS administration, although the lymphocyte numbers remained slightly reduced compared to baseline. As is known from literature,[14,21,22] circulating neutrophils mainly display a phenotype with decreased expression of CD16 or CD62L after LPS administration. Indeed, the percentage of $CD62L^{dim}$ and $CD16^{dim}$ neutrophils was increased after LPS administration compared to baseline (Figure 1E). This increase in $CD62L^{dim}$ and $CD16^{dim}$ neutrophils was significant at 3h and 6h post LPS administration, while the increase of $CD16^{dim}$ neutrophils was also significant at 9 hours (Figure 1F).

LPS STIMULATION ACTIVATES NEUTROPHILS

For the *in vitro* studies, whole blood of 6 healthy volunteers was stimulated with 2 ng/mL, 10 ng/mL or 100 µg/mL LPS or left unstimulated for 1 hour. Afterwards, flow cytometry was performed to measure expression of CD11b, CD62L, CD63 and CD64. Representative histograms of *in vitro* stimulated neutrophils are shown in Figure 2A. Expression of CD11b on neutrophils dose-dependently increased after LPS stimulation (Figure 2B). Unstimulated neutrophils had an CD11b MFI of 32.0 ± 5.5, increasing to 63.0 ± 13.0 after stimulation with 2 ng/mL LPS. This increase was even stronger after stimulation with 10 ng/mL and 100 µg/mL (80.0 ± 10.2 and 116.4 ± 14.3, respectively). In contrast to CD11b, CD62L expression decreased after LPS stimulation. The MFI of CD62L on unstimulated neutrophils was 29.1 ± 5.3, which significantly decreased to 3.8 ± 2.1, 4.7 ± 3.5, and 4.0 ± 2.0 after stimulation with LPS at 2 ng/mL, 10 ng/mL, and 100 µg/mL LPS, respectively. Expression of CD63 only significantly changed after LPS stimulation at 100 µg/mL (0.73 ± 0.44 for 100 µg/mL vs. 0.17 ± 0.06 for unstimulated), although the MFI values remained relatively low compared to the other markers.

CD64 expression increased significantly after LPS stimulation at 10 ng/mL and 100 µg/mL (4.2 ± 1.2 for 10 ng/mL and 5.2 ± 1.3 for 100 µg/mL vs 2.5 ± 0.4 for unstimulated).

The same flow cytometric analysis was performed on whole blood collected before (-15 min) and after (0.5, 3, 6, 9, 24h) i.v. administration of LPS (1 ng/kg) to 12 healthy volunteers. Representative histograms are shown in Figure 2C. Similarly to the *in vitro* stimulation experiment, CD11b expression significantly increased after LPS administration, peaking at 3 hours (MFI of 22.3 ± 11.6 at 3h vs. 10.9 ± 6.2 at baseline). The decrease in CD62L expression was also found *in vivo*, with the lowest expression found at 6h (27.61 ± 6.38) and 9h (27.60 ± 3.59) after LPS administration (baseline: 40.21 ± 12.18). Although only minor LPS effects were found on expression of CD63 and CD64 *in vivo*, these differences were significant at 3 hours post LPS administration (Figure 2D).

LPS STIMULATION INDUCES THE RELEASE OF NEUTROPHIL GRANULE PROTEINS

In addition to surface activation markers on neutrophils, neutrophil activation was measured by determining the protein excretion of MPO, elastase and LL37 via degranulation in plasma both after *in vitro* as well as *in vivo* LPS stimulation. Elastase levels were significantly increased after *in vitro* LPS activation at 2 ng/mL for one hour (2292 ± 827 ng/mL for 2 ng/mL vs. 892 ± 663 ng/mL for unstimulated whole blood) (Figure 3A). Elastase levels were further increased with increasing levels of LPS, 2558 ± 863 ng/mL for 10 ng/mL and 5833 ± 687 ng/mL for 100 µg/mL. Interestingly, levels of myeloperoxidase (MPO) and LL37 were only increased after stimulation with 100 µg/mL LPS (2737 ± 761 ng/mL for 100 µg/mL LPS vs. 439 ± 254 ng/mL for unstimulated and 65.6 ± 14.2 for 100 µg/mL LPS vs. 42.3 ± 8.1 ng/mL for unstimulated respectively). These effects are calcium-dependent, since LPS stimulation at 100 µg/mL in K_2EDTA anti-coagulated blood did not result in a strong increase in elastase, MPO or LL37 (Figure S2).

After *in vivo* LPS stimulation (2 ng/kg, 10 subjects), elastase levels were significantly increased compared to baseline, peaking at 6 hours (841 ± 1234 ng/mL at 6h vs. 98 ± 23 ng/mL at baseline, Figure 3B), despite a high variation between subjects. MPO levels were also highly variable between subjects, but again a significant increase was observed compared to baseline, peaking at 3h (109 ± 55 ng/mL at 3h vs. 22 ± 11 ng/mL at baseline). LL37 levels were less variable between subjects and peaked later than the other granule proteins, at 10 hours after LPS administration (108 ± 16 ng/mL at 10h vs. 57 ± 12 ng/mL at baseline).

DIFFERENTIAL EXPRESSED PROTEINS AFTER IN VIVO LPS ADMINISTRATION

To assess the impact of LPS on the neutrophil proteome, neutrophils were isolated and analyzed using shotgun proteomics before and 3 hours after *in vivo* LPS administration (2 ng/kg, n=4, Figure 4A). After LPS administration, only 4 proteins showed increased expression compared to baseline, while 22 proteins were decreased (Figure 4B). A table of all differentially expressed proteins is shown in Figure 4C, with the most significantly differentially expressed proteins listed first in the table. The proteins that are increased (14-3-3 protein gamma, protein transport protein SEC31A, beta-actin like protein 2 and 40S ribosomal protein SA) all play a role in regulation of the immune response[23,24] and intracellular protein synthesis and transportation. Of the decreased proteins (22 in total), 5 are normally stored in neutrophil granules. This decrease can therefore be attributed to degranulation of the cells upon activation. The other proteins are part of the cytoskeleton (keratins), play a part in the cell metabolism, are involved in signaling pathway modulation or are cytoplasmic proteins. It should be noted that keratins are notorious contaminants in proteomics and we cannot rule out that these differences stem from contamination.

Discussion

First, we aimed to provide a time course of neutrophil activation after *in vivo* LPS administration. A decline in lymphocytes and monocytes was seen in blood after i.v. LPS administration, while the number of neutrophils, and thereby the number of total leukocytes strongly increased. This is in line with literature.[12,25-27] As shown before,[14,21,22] the neutrophils mainly display a $CD16^{dim}$ (banded) and $CD62L^{dim}$ (hypersegmented) phenotype, in contrast to homeostasis where neutrophils are mainly of a $CD16^{hi}CD62L^{hi}$ phenotype. For the cause of this decrease in CD62L expression, it is impossible to distinguish between activation and subsequent shedding of CD62L, or influx of $CD62L^{dim}$ neutrophils. The peak of neutrophil counts and the lowest expression of CD62L *in vivo* coincide at 6 hours after LPS administration (Figure

S4), indicating that this decrease in CD62L expression is likely due to influx of neutrophils from the bone marrow. Besides CD62L, CD11b was investigated as a marker of activation on neutrophils.[18,19] A rapid increase in CD11b expression was observed peaking at 4 hours after LPS administration. Also, the expression of CD63 and CD64 were significantly increased after *in vivo* LPS exposure, peaking at 4 hours, although this increase was only moderate. CD63, also known as lysosome-associated membrane protein (LAMP3), is part of the tetraspanin family. In neutrophils CD63 is expressed on membranes of primary granules, controlling granule sorting of proteins such as neutrophil elastase.[28,29] Therefore, increased cell surface expression can be used as a marker of neutrophil degranulation.[30] It has been shown *in vitro* that aging neutrophils increase the surface expression of CD63, and surface CD63 expression was restricted to the apoptotic neutrophil population.[31] CD64 (Fcγ receptor I) is not constitutively expressed on neutrophils, but can be induced upon stimulation. CD64 expression on neutrophils is a marker for sepsis,[32,33] and a trial in which healthy volunteers received 2 ng/kg LPS showed an increase in CD64 neutrophil expression upon endotoxemia.[34] Coinciding with the expression of CD63 and CD64, azurophilic granule proteins MPO, elastase and LL37[35] were rapidly increased after LPS administration. Interestingly, at the level of the neutrophil proteome, little but significant differences were observed in protein expression at 3 hours after LPS administration compared to baseline. These differences point to neutrophil degranulation. However, changes in protein expression were not as prominent as expected for the massive neutrophil activation and degranulation indicated by the experiments discussed above. In this context, it is important to note that activation markers such as receptors are already contained in the granules. Upon cell activation, they are transported to the cell membrane, and the exact cellular location does not impact the total neutrophil proteome. Additionally, activation of neutrophil receptor relocalization leads to enhanced adhesion of neutrophils on the blood vessel and subsequent degranulation. These neutrophils are not present and analyzable in blood samples. As shown previously by de Kleijn et al., significant changes in the transcriptome of neutrophils were found in human experimental endotoxemia, mostly due to neutrophil activation by inflammatory cytokines.[27]

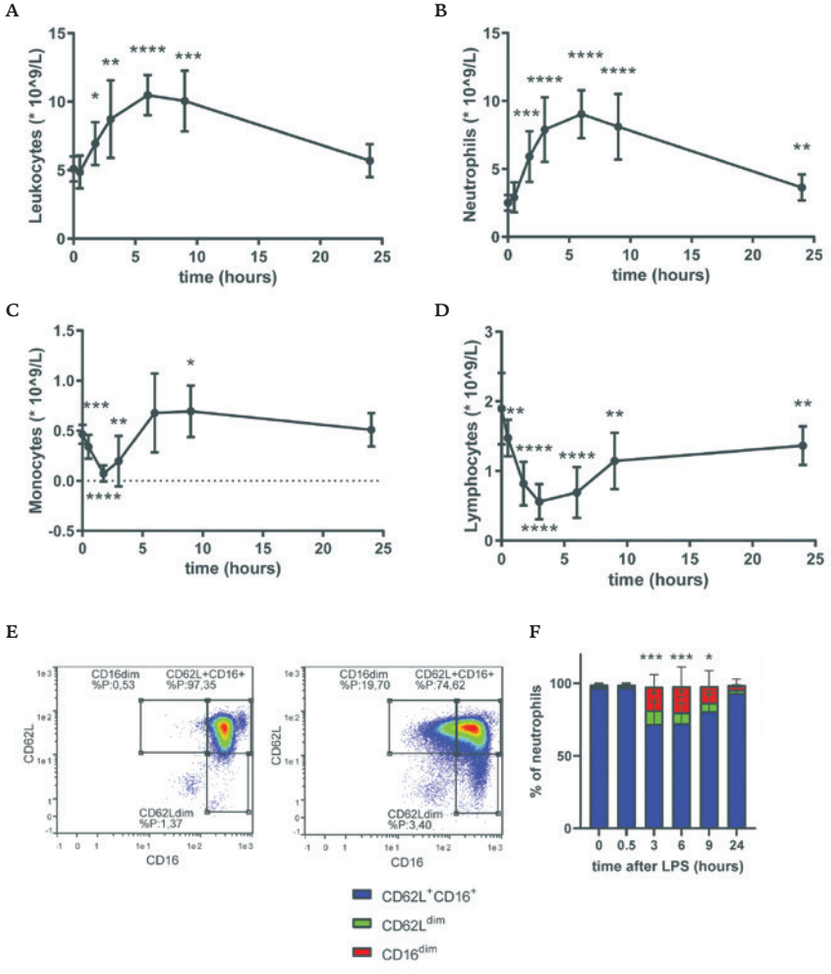
When comparing the *in vivo* and *in vitro* LPS-driven neutrophil responses, some differences can be observed. First of all, the dose of LPS required for neutrophil activation is higher in *in vitro* experiments. While *in vivo* a dose of 1 or 2 ng/kg of LPS drove significant neutrophil responses, corresponding to <0.05 ng of LPS per mL of blood depending on the size/weight of the volunteer, *in vitro* higher concentrations (ranging from 2 ng/mL to 100 µg/mL) were necessary to measure effects. This discrepancy in required trigger concentration can be explained by the fact that in whole blood cultures only direct effects of LPS on leukocytes are studied, while *in vivo* other factors play a role, such as general inflammation of the endothelium of the vessel wall.[36,37] Another factor complicating comparison of *in vitro* and *in vivo* results is the above discussed effect that as soon as neutrophils get activated, they will adhere to the vessel wall and thereby will not be analyzed after blood draw. This could also explain the relatively limited increase in neutrophil activation markers, for instance CD11b, upon *in vivo* LPS exposure. While *in vitro* more LPS was needed to activate neutrophils, the LPS-driven expression of activation markers CD62L and CD11b was much more pronounced compared to *in vivo.* In contrast to activation markers, granule protein plasma levels are clearly increased upon *in vivo* LPS stimulation in line with the *in vitro* data. Interestingly, the baseline levels of elastase and MPO in the *in vivo* study were lower than the unstimulated control in the *in vitro* experiments. This would mean that an hour of whole blood incubation already triggers a low level of degranulation although this effect was not seen for LL37. Furthermore, MPO and LL37 levels were only significantly increased at the highest LPS concentrations, indicating that low levels of LPS alone is not sufficient to induce high level degranulation, and that additional activation triggers are needed to activate neutrophil degranulation *in vivo*, such as interaction with the endothelium.

In general, *in vitro* and *in vivo* activation of neutrophils by LPS were quite comparable when looking at activation markers and granule proteins. Although the minimal LPS concentration required to drive neutrophil responses, and the neutrophil response sizes, differed upon *in vivo* and *ex vivo* LPS exposure, the nature of the observed responses were well comparable between the *in vivo* and *in vitro* challenge models. Based on these data, both the *in vitro* whole blood LPS stimulation assay and the human endotoxemia model could be valuable tools for evaluation of the effects of future drugs modulating neutrophil responses, both during preclinical and clinical development.

Table 1 Demographics of the different studies.

Study	Age, years Median (Q25–Q75)	Gender (% male)	Ethnicity (% white)	BMI kg/m^2 median (Q25–Q75)
In vivo, 1 ng/kg (n=12)	25.0 (23.0–30.75)	91.7	91.7	25.05 (21.15–26.23)
In vivo, 2 ng/kg (n=10)	25.0 (23.0–32.5)	100	50	24.75 (21.43–25.65)
In vitro, flow cytometry exp (n=6)	37.5 (30.5–40.75)	16.7	83.3	N/A
In vitro, granule protein exp (n=6)	25.0 (22.75–42.50)	50	100	N/A

Figure 1 Circulating leukocyte levels after in vivo LPS administration.



*Blood was collected before (-15 min) and after (0.5, 1.75, 3, 6, 9, 24, 48h) LPS administration (1 ng/kg, n=12). The number of leukocytes (A), neutrophils (B), monocytes (C) and lymphocytes (D) was determined by hematocytometry. Total neutrophils were analyzed for CD16 and CD62L expression (E), where an increase in CD62L^{dim} and CD16^{dim} neutrophils was found (F). Mean and SD are shown. Statistics was performed using 1-way repeated-measures analysis of variance with Dunnett's post hoc test, where post-dose time points were compared to t=0h. * P≤0.05, ** P≤0.005, *** P≤0.001, **** P≤ 0.0001.*

Figure 2 Neutrophil activation markers CD11b, CD62L, CD63 and CD64. Whole blood of 6 subjects (1 male, 5 female, median age 37.5 years, 2 separate experiments) was stimulated with 2 ng/mL, 10 ng/mL, 100 µg/mL or no LPS for 1 hour. CD11b, CD62L, CD63, CD64 expression on neutrophils was measured by flow cytometry. Representative histograms of 1 subject are shown (A). MFI is shown in panel (B). CD11b, CD62L, CD63, CD64 expression on neutrophils of 12 subjects receiving 1 ng/kg LPS was measured by flow cytometry at -1h prior to dosing up until 24 hours LPS administration. Representative histograms of 1 subject are shown (C), mean with SD is shown in (D). Statistics for panel B was performed using one-way ANOVA with Dunnett's post hoc test, statistics for panel D was performed using 1-way repeated-measures analysis of variance with Dunnett's post hoc test, where post dose time points were compared to t=-1h. * P≤0.05, ** P≤0.005, *** P≤0.001, **** P≤ 0.0001.

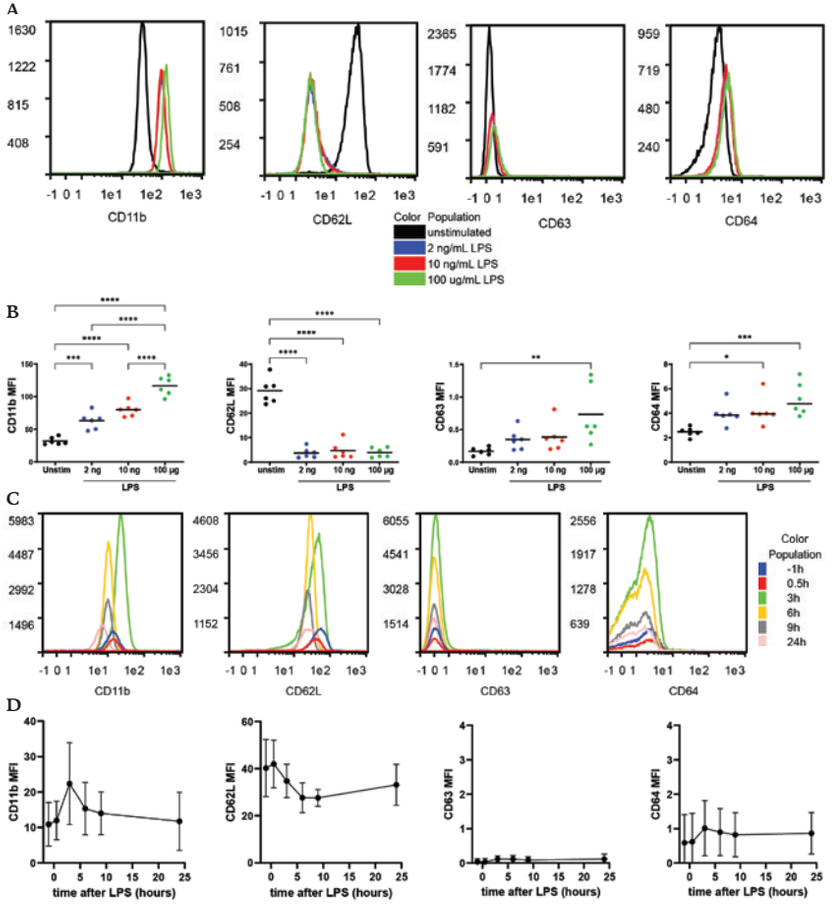


Figure 3 Granule protein measurements. Whole blood of 6 subjects (3 male, 3 female, median age 25 years) was stimulated with 2 ng/mL, 10 ng/mL, 100 µg/mL or PBS control (Unstim) for 1 hour in 2 separate experiments. Supernatants were collected and elastase, MPO and LL37 levels were determined by ELISA (A). Samples before (-1h) and after (1h, 2h, 3h, 4h, 6h, 10h and 24h) LPS administration (2 ng/kg, n=10) were assayed for elastase, MPO and LL37 levels by ELISA (B). Statistics for panel A was performed using one-way ANOVA with Dunnett's post hoc test, statistics for panel B was performed using 1-way repeated-measures analysis of variance with Dunnett's post hoc test, where post dose time points were compared to t=-1h. * P≤0.05, ** P≤0.005, *** P≤0.001, **** P≤ 0.0001.

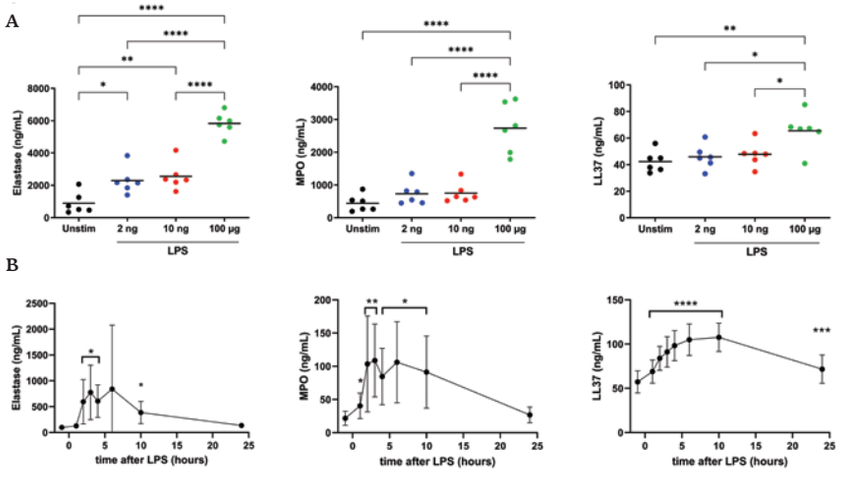
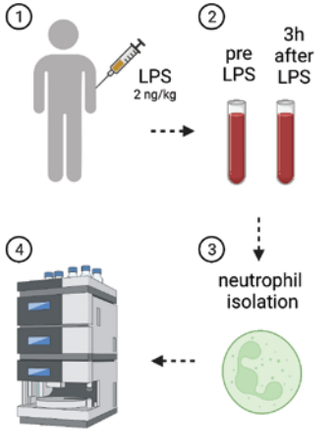


Figure 4 Proteomics of neutrophils before and after in vivo LPS stimulation.
Blood was drawn from subjects receiving 2 ng/kg LPS (n=4), before and 3 hours after LPS administration, and neutrophils were isolated. Shotgun proteomics was performed with these isolated neutrophils (A). Differentially expressed proteins are visualized in a volcano plot (B). A table with all significantly increased and decreased proteins in included in (C). Panel A was made using biorender.com.

A



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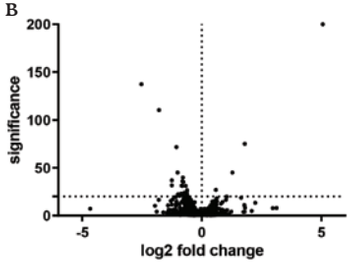


C

Increased
14-3-3 protein gamma
Protein transport protein SEC31A
Beta-actin-like protein 2
40S ribosomal protein SA
Decreased
Hornerin
Keratin type I cytoskeletal 9
Keratin type I cytoskeletal 10
Phosphatidylethanolamine-binding protein 1
Peptidoglycan recognition protein 1
Complement C3
Protein S100-P
Adenylyl cyclase-associated protein 1
Keratin type II cytoskeletal 1
Transaldolase
Keratin type II cytoskeletal 2 epidermal
Lysozyme C
Neutrophil elastase
Malate dehydrogenase cytoplasmic
Vascular non-inflammatory molecule 2
Fructose-bisphosphate aldolase C
Fatty acid-binding protein 5
Cysteine-rich secretory protein 3
Ubiquitin-conjugating enzyme E2 N
Chitotriosidase-1
Cytochrome P450 4F3
Succinate--CoA ligase [GDP-forming] subunit beta

Supplemental material

Table S1 Flow cytometry antibody list.

Marker	Fluorochrome	Clone	Supplier
CD11b	VioBlue	REA713	Miltenyi
CD14	FITC	REA599	Miltenyi
CD16	BV605	3G8	Biolegend
CD45	VioGreen	REA747	Miltenyi
CD62L	PE	REA615	Miltenyi
CD63	APC	REA1055	Miltenyi
CD64	PERCP-VIO700	REA978	Miltenyi
CD66b	PE-VIO770	REA306	Miltenyi

PE=Phycoerythrin, BV=Brilliant Violet, FITC=Fluorescein isothiocyanate, APC=allophycocyanin, PERCP=Peridinin-Chlorophyll-Protein

Figure S1 Gating strategy neutrophil activation markers. First all cells are gated in the FSC-A/SSC-A plot, excluding debris. Then singlets are gated in the FSC-A/FSC-H, followed by CD45^{+} PI- live cells. Then granulocytes are gated in the FSC-A/SSC-A plot, followed by neutrophils, gated as CD16^{+}CD66b^{+}. From the neutrophil gate expression of CD62L, CD63, CD64 and CD11b is measured.

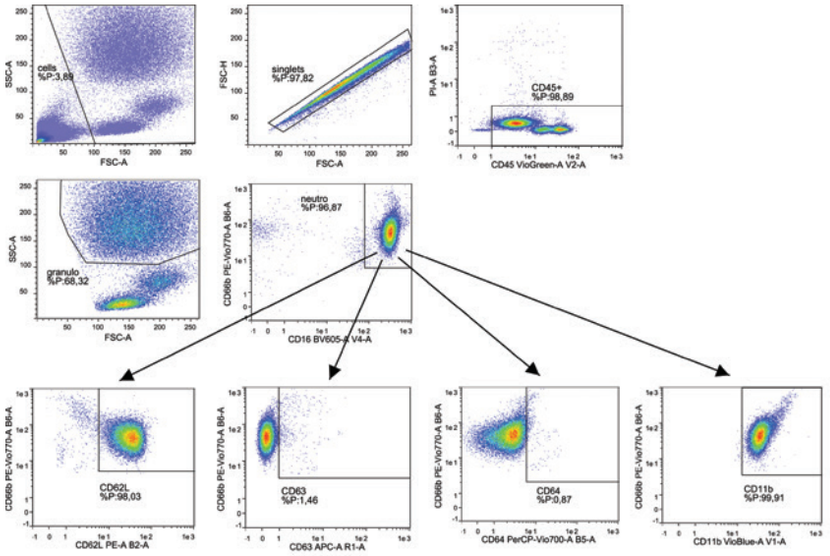


Figure S2 LPS stimulation in K_2EDTA whole blood. K_2EDTA anticoagulated whole blood was stimulated with 100 µg/ml LPS or PBS control for 1 hour. Supernatant was assayed for elastase, MPO and LL37 (n=3 for elastase and LL37, n=2 for MPO).

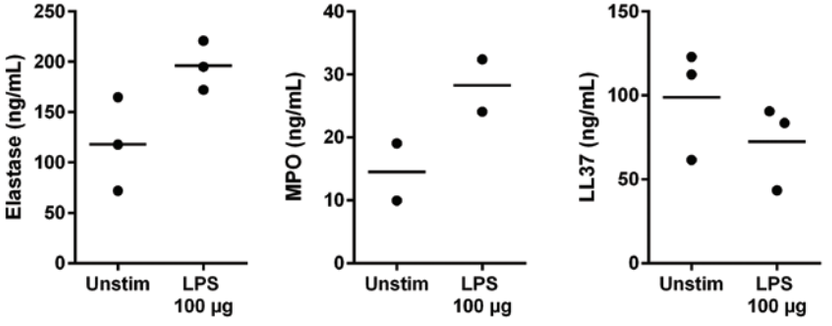


Figure S3 CD62L and CD16 expression on neutrophils, *in vitro*. CD16 and CD62L expression on unstimulated (left) and 100µg/mL LPS stimulated neutrophils (right).

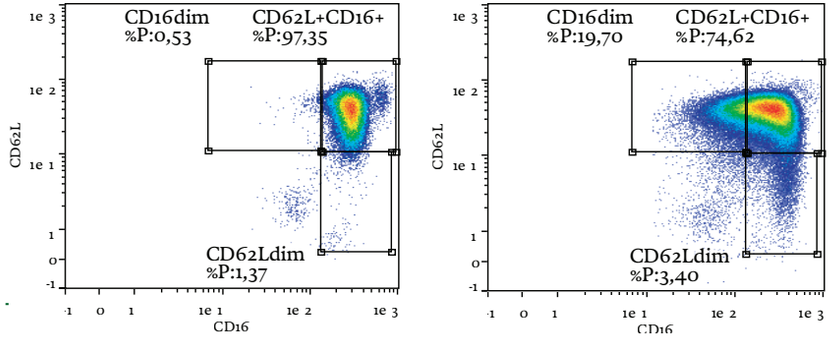
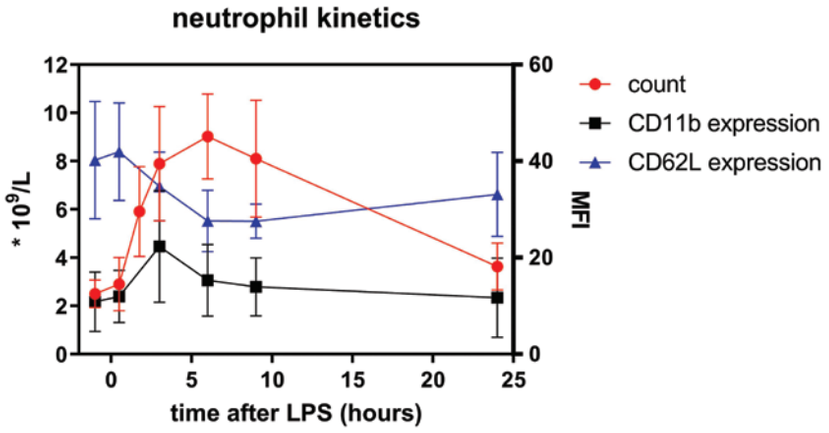


Figure S4 Neutrophil kinetics *in vivo*. The neutrophil count (red), CD11b MFI (black) and CD62L MFI (blue) over time after LPS administration.



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CHAPTER 4

Whole blood assay as a model for in vitro evaluation of inflammasome activation and subsequent caspase-mediated interleukin-1 beta release

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Graphical abstract

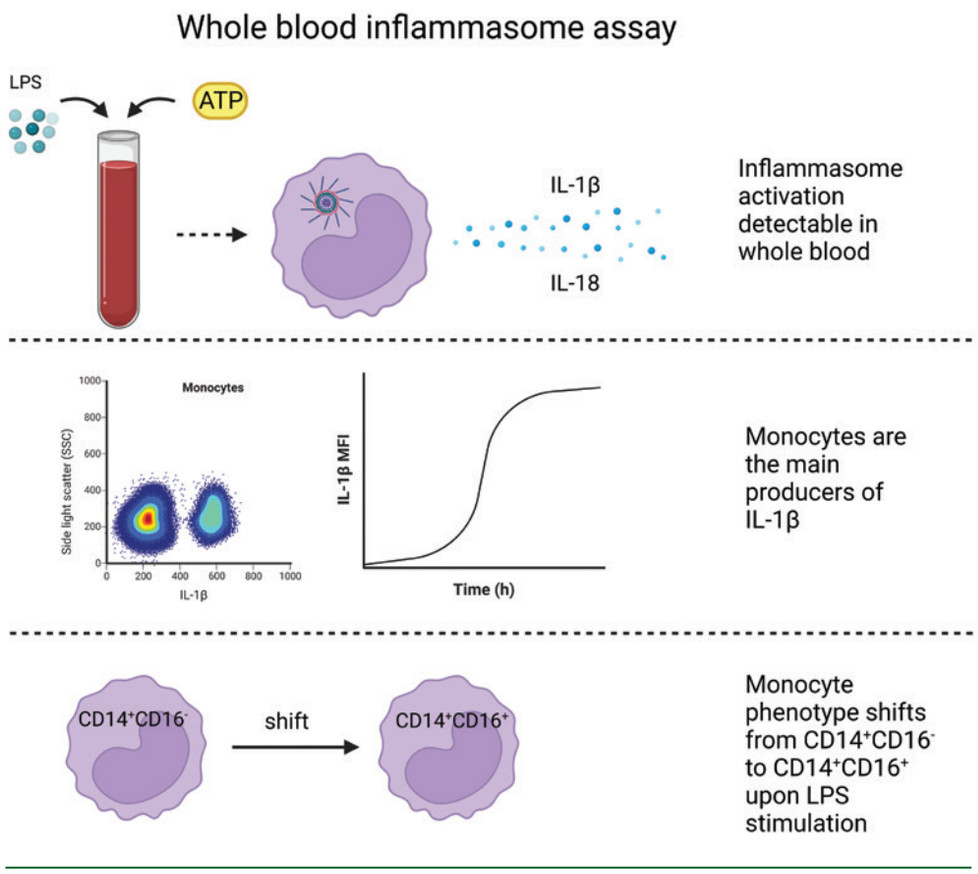


Figure is created using biorender.com.

Abstract

Processing of pro-interleukin (IL)-1β and IL-18 is regulated by multiprotein complexes, known as inflammasomes. Inflammasome activation results in generation of bioactive IL-1β and IL-18, which can exert potent pro-inflammatory effects. Our aim was to develop a whole blood-based assay to study the inflammasome *in vitro* and that also can be used as an assay in clinical studies. We show whole blood is a suitable milieu to study inflammasome activation in primary human monocytes. We demonstrated that unprocessed human blood cells can be stimulated to activate the inflammasome by the addition of adenosine 5'-triphosphate (ATP) within a narrow timeframe following lipopolysaccharide (LPS) priming. Stimulation with LPS resulted in IL-1β release; however, addition of ATP is necessary for "full-blown" inflammasome stimulation resulting in high IL-1β and IL-18 release. Intracellular cytokine staining demonstrated monocytes are the major producers of IL-1β in human whole blood cultures, and this was associated with activation of caspase-1/4/5, as detected by a fluorescently labelled caspase-1/4/5 probe. By applying caspase inhibitors, we show that both the canonical inflammasome pathway (via caspase-1) as well as the non-canonical inflammasome pathway (via caspases-4 and 5) can be studied using this whole blood-based model.

Introduction

Inflammasomes are large multimolecular complexes controlling the activation of caspase-1,[1]in response to bacterial and damage-associated stimuli.[2,3] One of the most studied inflammasome members is nucleotide binding and oligomerization domain and leucine rich-repeat-containing pyrin domain containing 3 (NLRP3).[4] The NLRP3 complex formation by sequential triggers is known as a canonical pathway of inflammasome activation. The first stimulus initiates nuclear factor-κB (NF-κB) activity by Toll-like receptor (TLR) signaling, thereby inducing IL1B and IL18 mRNA synthesis[5] and licensing the expression of the NLRP3 inflammasome.[6] The second stimulus (for example ATP, potassium efflux, release of mitochondrial DNA, lysosomal damage) leads to the oligomerization and formation of the NLRP3 protein complex, resulting in caspase-1 activation and consequently release of mature IL-1β and IL-18.[7–9] A so-called 'non-canonical' pathway has been identified,[10] comprising caspase-11 in mice and caspase-4/5 in humans,[11–13] which may produce IL-1β but not IL-18 after a single stimulus. The inflammasome activation is also regulated by caspase-8 that participate as a modulator of canonical NLRP3 signaling[14] or as a trigger of non-canonical IL-1β processing.[15]

Mature IL-1β is involved in upregulation of adhesion molecules, induction of chemokines and infiltration of immune cells into tissues.[1] IL-1β–induced pro-inflammatory responses activate host defense during infection while IL-18 drives interferon-gamma (IFNγ) expression in T cells and NK cells.[16] Upregulation of IL-1β expression is observed in multiple disorders including Alzheimer's disease, diabetes mellitus, atherosclerosis and hypertension[17–19] whereas elevated levels of circulating IL-18 have been reported for patients with heart disease[16] and a role for IL-18 has been suggested in various autoimmune diseases.[16,20,21] Given the effector functions of IL-1β and IL-18 and their reported enhanced expression in various pathological conditions, the inhibition of NLRP3 activation or its products may offer a valuable therapeutic approach. The early clinical testing of such agents is hindered by the fact that although IL-18 is constitutively present in plasma,[22] NLRP3 activity and IL-1β levels are only increased after stimulation, so assessment of inflammasome inhibition in healthy volunteers is not straightforward. However, NLRP3 inflammasome activity can be induced *ex vivo* in primary human cells, and such models can be employed as pharmacodynamic readout in (pre)clinical pharmacology studies. We previously described the application of caspase-1 inhibitors for inflammasome assays in human whole blood.[23] Other research groups commonly use peripheral blood mononuclear cell (PBMC)-based models or differentiated macrophages when investigating (modulation of) inflammasome activity.[24–27] The secretion of pro-inflammatory cytokines in whole blood in conditions where inflammasome activation is expected has been investigated in the past[28–30] and large differences in variables such as assay matrices, inflammasome triggers, readouts and incubation durations have been reported.

In this study, we thoroughly evaluated the kinetics of inflammasome activation in whole blood, we explored the dynamics of inflammasome stimulation by parallel cytokines, we investigated the potential occurrence of cell death or factors inhibiting inflammasome activity, and we identified by flow cytometry the cell types that are accountable for IL-1β production. We validated our whole blood-based model by application of specific caspase inhibitors, differentiating between the canonical pathway (caspase-1) and the non-canonical pathway (caspase-4/5).

Materials and methods

BLOOD COLLECTION

Venous blood was collected from healthy volunteers into sodium-heparin tubes (Becton Dickinson, San Jose, CA, USA) after obtaining written informed consent in accordance with relevant guidelines and regulations. The protocol was approved by the Medical Ethical committee of Leiden University Medical Center.

EX VIVO WHOLE BLOOD CULTURE & STIMULATION

Cell cultures were performed in an endotoxin-free manner. The blood was stimulated 1:1 volume with either vehicle (CTRL), 2 ng/ml LPS (*Escherichia coli* serotype 0111:B4) or in combination of LPS with ATP (5mM) in duplicate. LPS and ATP were both obtained from Sigma-Aldrich (St Louis, MO, USA). All stimulants were diluted in RPMI 1640 (Gibco, Life technologies) and samples were incubated at 37°C, 5% CO_2 for 4, 8, 12 and 24h with LPS or LPS+ATP where the ATP was added for the last 30 min of the incubation time. Baseline sample (CTRL 0h) without stimulation 1:1 volume in RPMI 1640 was also

processed. NLRP3 inhibition and caspase inhibition was performed with 1h pre-incubation with MCC950 (5 µM), AC-LEVD-CHO and AC-YVAD-CMK for the indicated concentrations prior to inflammasome stimulation with LPS for 3.5h and ATP for an additional 30 min more. MCC950 is a potent and selective inhibitor of NLRP3 (Invivogen), AC-LEVD-CHO inhibits caspase-4 and 5 (Enzo Lifesciences) and AC-YVAD-CMK targets caspase 1, 4 and 5 (Sigma-Aldrich). Following incubation, the supernatants of whole blood cultures were collected after centrifugation 2000g for 20 min. Obtained supernatants were frozen on dry ice for 15 min and stored at -80°C for later cytokine analysis. The cells were further processed for flow cytometry analysis.

PRO-INFLAMMATORY CYTOKINES ANALYSIS

IL-1β levels from blood culture supernatants were determined by the Meso Scale Discovery platform (human proinflammatory V-Plex plus Kit, MSD,) or Quantikine ELISA (R&D Systems) as indicated in the figure legends. The supernatant levels of tumor necrosis factor alpha (TNFα), IL-6 and IL-8 were determined with the Human Proinflammatory 4-Plex II Ultra-sensitive Kit (Meso Scale Discovery, MSD). IL-18 was measured by ELISA (MBL) or MSD, as indicated in figure legend. IL-18BPA and IL-1SRII levels were determined by ELISA (R&D Systems). ASC/PYCARD was measured by ELISA (Aviva Systems Biology). All measurements were performed according to manufacturer's instructions.

FLOW CYTOMETRY

Following stimulation, 100 µl whole blood sample of each condition (CTRL, LPS, LPS+ATP) was used for flow cytometry. Red blood cells were lysed using 2 ml RBC lysis buffer (Biolegend), incubated at room temperature for 10 min, followed by centrifugation at 350g for 5 min. The samples were washed twice in FACS buffer (PBS + 0.5% BSA) and stained with 5 µl of the following antibodies anti-CD3-FITC (clone OKT3), anti-CD14-FITC (clone M5E2), anti-CD20-FITC (clone 1412), anti-CD15-PE/CY7 (clone W6D3), anti-CD16-APC/CY7 (clone 3G8), anti-CD45-pacific blue (clone HI30) (all from Biolegend), anti-CD20-APC (clone 2H7, eBioscience), 1 µl anti-CD56-APC (clone CMSSB, eBioscience), 1 µl anti-HLA-DR-PERCY5.5 (clone G46-6, Becton Dickinson), CD123-PE (clone 9F5, BD) and 1 µl CD11C-FITC (clone B-LY6, BD) by incubation for 30 min at 4°C in the dark. To check for cell viability an APC Annexin V Apoptosis Detection Kit with PI (Biolegend) was used following manufacturer's instructions. Intracellular IL-1β and ASC staining were done in the presence of a protein transport inhibitor cocktail containing both brefeldin A and monensin (eBioscience). The samples were fixed and permeabilized with fixation buffer (BioLegend) and intracellular permeabilization wash buffer (Biolegend), respectively. Blocking was done with Human TruStain FcX (FC Receptor Blocking Solution) (Biolegend) and then samples were stained with 5 µl IL-1β-PE antibody (clone CRM56, eBioscience) and 1 µl ASC (TMS1)-AF647 (MBL International) for 30 min at 4°C in the dark. Matched isotype controls were used from Biolegend, eBioscience and MBL International. FLICA, FAM-YVAD-FMK, (Immunochemistry technologies) staining was done after red blood cell lysis and surface staining for CD14 was done with anti-CD14-APC (clone M5E2, Becton Dickinson) according to manufacturer's instructions. The cells were analyzed on a MACSQuant Analyzer 10 flow cytometer (Miltenyi Biotec). Data was analyzed using FlowJo software version 10 (FlowJo LLC). Representative dot plots of gating strategy for assessment of IL-1β expression in monocytes and neutrophils (S1 Fig panel A), for monocyte phenotype determination (S1 Fig panel B) and for evaluation of IL-1β$^+$ cells among monocytes and dendritic cells (S1 Fig panel C).

STATISTICAL ANALYSIS

Data are presented as mean value ± standard error of the mean (SEM). Differences in IL-1β release and expression were analyzed by two-way ANOVA and mixed effects model using Tukey's test for multiple comparisons, respectively. Differences in monocyte marker expression at different time-points were analyzed by mixed effects analysis with Dunnett's multiple comparison test. Differences was considered significant if $P<0.05$. For statistical analysis GraphPad Prism version 8.00 software for Windows (GraphPad Software, La Jolla, CA, USA) was used.

Results

IL-1β AND IL-18 RELEASE IN WHOLE BLOOD

To compare NLRP3 inflammasome activation of minimally processed whole blood, samples were incubated for 4, 8, 12 and 24h, with LPS (full incubation duration), or LPS (full incubation duration) plus ATP (LPS+ATP; final 30 min of the incubation time). After 4h of incubation with LPS an increase in IL-1β was seen, which was accentuated upon addition of ATP for the final 30 min

of the incubation period in six donors (Fig 1A). Similar pattern of production was observed for IL-18 in one donor (S2 Fig). Substantial levels of TNFα and IL-6 was detected upon the addition of ATP following LPS priming at 12h, while IL-8 did not result in substantial changes to secretion with the addition of ATP. Elevated levels of TNFα and IL-6 were observed at 12h incubation, thus it is unlikely that these two cytokines are involved in release of IL-1β and IL-18 in the supernatant (S2 Fig). After 24h of incubation the release of IL-1β and IL-18 was generally lower after addition of ATP compared to earlier time points (Fig 1A).

The fact that ATP-induced IL-1β and IL-18 release peaked after 4h of LPS priming and decreased upon extended priming suggests that either a loss of specific cell populations occur, or some inhibiting factors may be rapidly up-regulated to counter the responses induced by LPS+ATP. To explore if the loss of specific cell populations might explain the decrease of IL-1β and IL-18 secretion, cell viability was assessed by measuring the number of monocytes and neutrophils in cultures as well as by annexin V/PI staining. The number of viable monocytes in whole blood cultures was slightly decreased at 4 and 8h for stimulated samples while it decreased at the end of incubation period for control as well as stimulated samples (Fig 1B). At 4 and 8h a decrease of the neutrophil population is observed for the stimulated conditions but not the control, while this population is restored at 24h (Fig 1C). Even though both monocytes and neutrophils cell numbers decreased during stimulation no statistically significant differences were observed. Furthermore, the staining with annexin V showed that still 75–100% of the monocytes and neutrophils were viable during the 24h experimental procedures (Fig 1D and 1E).

To test the second hypothesis whether inhibiting factors may dampen the whole blood responses induced by LPS+ATP, soluble IL-1 receptor II (IL-1SRII) and IL-18 binding protein a (IL-18BPA) levels were measured in supernatants of whole blood cultures, and none of these negative regulators exhibited changes coinciding with observed IL-1β and IL-18 responses, (S3 Fig).

MONOCYTES ARE A MAJOR SOURCE OF IL-1β

IL-1β is a potent pro-inflammatory cytokine produced by cells of the innate immune system. The IL-1β production in innate immune cells in whole blood was performed by co-staining for intracellular IL-1β and for cell surface markers used for characterization of monocytes, neutrophils and dendritic cells as explained in materials and methods. The pattern of IL-1β induction in $CD14^+$ monocytes resembles the release of IL-1β in whole blood over a period of 24h, with statistically significant increase of IL-1β in LPS and LPS+ATP compared to unstimulated control at 4 and 8h (Fig 2A), indicating that monocytes are a major contributor to the responses in whole blood to LPS and LPS+ATP. Flow cytometry evaluation of IL-1β expression in $CD15^+CD16^+$ neutrophils showed that inflammasome activating condition (LPS+ATP) did not induce significant IL-1β compared to control or LPS stimulated whole blood samples (Fig 2B), while myeloid dendritic cells to a minor extend contribute to IL-1β processing in whole blood assay (S4 Fig).

NLRP3 INFLAMMASOME INVOLVEMENT IN IL-1β GENERATION

To elucidate the role of NLRP3 the cells were stained with apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) together with markers for characterization of monocytes and neutrophils. At 4h after stimulation the monocytic cell population showed slight increase of ASC expression in LPS+ATP stimulated cells compared to control with further increase at 8h (Fig 3A). A different profile of ASC expression was observed for the neutrophils, with high levels at 8h in both control as well as LPS or LPS+ATP stimulated samples (Fig 3B). And for both population at 24h there were almost none detectable levels (Fig 3A and 3B). Additionally, release of ASC into the supernatant was also tested with detectable levels in stimulated conditions at 8h (3000 pg/ml) and further up-regulated 4-fold at 24h (12000 pg/ml) (Fig 3C). To further investigate the involvement of NLRP3 the well-known inhibitor MCC950 was applied only at the 4h condition, where a significant inhibitory effect of 65% was observed for the release of IL-1β (S5A Fig) and 90% for the release of ASC/PYCARD (S5B Fig) compared to the LPS+ATP condition.

MONOCYTES DISPLAY PHENOTYPE SHIFT UPON INFLAMMASOME ACTIVATION IN WHOLE BLOOD CULTURES

Human blood monocytes can be classified into three distinct populations, classical $CD14^+CD16^-$ monocytes, intermediate $CD14^+CD16^+$ and non-classical $CD14^{dim}CD16^+$ monocytes.[31] Each class contributes to the inflammatory response in different manners due to differences in expression of TLRs and cytokine/chemokine responses.[32] Flow cytometry analysis in basal, not

manipulated whole blood confirmed the existence of all three monocytic subsets with distribution similar to previous reports (S6 Fig).[33] Treatment with LPS or LPS+ATP did not induce prominent changes in CD14 and CD16 expression in all three subsets after 4h compared to control (Fig 4). At 8h the only change observed was of the $CD14^{+}CD16^{-}$ cell phenotype significantly decreased in both stimulated conditions (LPS and LPS+ATP) but not in the control (Fig 4A). And two subsets of the monocytes changed after 24h, namely the number of $CD14^{+}CD16^{-}$ cells (Fig 4A) declined, which was accompanied by the increase of $CD14^{+}CD16^{+}$ cells (Fig 4B) at the same time-point (see also S7 Fig). The number of $CD14^{dim}CD16^{+}$ cells was unaltered during the 24h experimental period. Intracellular staining for IL-1β in two donors demonstrated that $CD14^{+}CD16-$ are predominant source of IL-1β within the monocytic subsets (S8 Fig).

IL-1β AND IL-18 RELEASE IS INHIBITED BY CASPASE INHIBITORS

Upon confirmation that monocytes produce IL-1β, caspase-1/4/5 activity was investigated in this cell population using FAM-YVAD-FMK, a fluorescent probe that binds active caspase-1/4/5, referred to here as FLICA. $CD14^{+}$ monocytes were analyzed by flow cytometry after whole blood stimulation with LPS or LPS+ATP. After stimulation for 4h, LPS induced 12% ± 2% FLICA+ monocytes, while LPS+ATP treatment induced 31% ± 15% $FLICA^{+}$ monocytes (Fig 5B). In a separate experiment, stimulation for 1h showed only FLICA positive monocytes after LPS+ATP exposure (Fig 5A). These data demonstrate clearly caspase activation by monocytes in whole blood cultures and indicates that caspase-1 is not constitutively active in monocytes.

To confirm that in our whole blood cultures the intracellular IL-1β cleavage occurs as result of caspase activation after inflammasome assembly, we performed a whole blood experiment with caspase inhibitors AC-YVAD-CMK and AC-LEVD-CHO. Whole blood was incubated for 1h with 4, 20 or 100 μM AC-YVAD-CMK (preferential caspase-1 recognition site) or AC-LEVD-CHO (preferential caspase-4 recognition site) prior to inflammasome stimulation.[34] Following incubation with the caspase inhibitors, cultures were stimulated with LPS+ATP in order to specifically activate caspase-1.[35,36] Both inhibitors inhibited IL-1β release after LPS+ATP stimulation (Fig 6A) and IL-18 release after LPS+ATP stimulation (Fig 6B), showing caspases-1 and 4/5 were activated after stimulation in whole blood. These results were consistent in five additional blood cultures from healthy donors (S9A, S9B and S9C Fig). The fact that both inhibitors inhibited IL-1β and IL-18 release after inflammasome stimulation indicates that both caspase-1 and caspase-4 play a role in the processing of these cytokines. The effect of both inhibitors on TNFα and IL-6 release after LPS and LPS+ATP was measured as a control (S9D Fig), and here no effect of the inhibitors was observed.

Discussion

Altered inflammasome activity has been implicated in multiple diseases including pseudogout, asbestosis, Alzheimer's, cancer, atherosclerosis, and type 2 diabetes mellitus.[19,27,37] In order to set up an easy and robust assay for inflammasome research, we explored the use of *ex vivo* whole blood cultures as a tool to study inflammasome activation resembling the *in vivo* circumstances as much as possible. The isolation of PBMCs or other cells, such as macrophages from peripheral blood, may induce unwanted side effects such as a higher level of apoptosis in isolated PBMCs compared to whole blood.[38] Moreover, in whole blood cultures the influence of sample handling-related cell activation is minimal, so this is the most natural milieu to study cytokine production *in vitro*.[39]

First, we optimized our conditions by evaluating the optimal time-frame for inflammasome activation. After priming human whole blood with LPS for periods up to 24h, subsequent co-stimulation with ATP did not result in increased secretion of IL-1β and IL-18 as compared to shorter LPS incubations (4 and 8h). Evaluation of the secretion of potential inhibitory mechanisms such as IL-1SRII and IL-18BPA did not demonstrate any related kinetic patterns associated with limited time-frame of increased IL-1β production, suggesting these are not critical factors in the down-regulation of IL-1β. To further investigate what underlies IL-1β and IL-18 decrease at 24h, we studied the effect of LPS priming and ATP pulse application on viability of monocytes and neutrophils. Although the number of $CD14^{+}$ monocytes declined for certain conditions, yet the viability of cells is not significantly affected as measured by staining for annexin V/PI. Similar results were obtained for $CD15^{+}CD16^{+}$ neutrophils indicating that cell death is not a reason for reduction of IL-1β. Preserved cell viability in our experimental setting is based on use of low LPS and ATP concentrations (2 ng/mL LPS + 5 mM ATP) as compared to studies that routinely have

used higher concentrations (1–2 μg/mL LPS + 1–5 mM ATP).[40,41] This is in accordance with previously reported cell viability in similar settings.[40,42,43] Taken together, priming cells with LPS for 24h and incubating with ATP for 30 min results in diminished IL-1β and IL-18 induction indicating that a negative feedback mechanism is mainly responsible for the observed time-response courses in our model. This is also in line with the growing paradigm for negative feedback initiated by LPS signaling patterns in general.[44–46] In addition, a recent study of Gurung et al. has identified IL-10 as a soluble secreted factor that acts as a negative feedback loop to dampen NLRP3 inflammasome activation.[47]

It is well acknowledged that monocytes are major source of IL-1β in circulation although recently it has been shown that neutrophils also contribute to the total pool of circulating IL-1β.[48] Our data indeed suggests that the majority of IL-1β is produced by monocytes and neutrophils. Detectable intracellular MFI levels of IL-1β in neutrophils irrespective of stimulation suggests that even a minimal manipulation of whole blood might trigger neutrophil activation, followed by increase of intracellular IL-1β levels or that neutrophils are constitutively loaded with IL-1β. Inflammasome activation in whole blood was confirmed by FLICA staining but evaluation of the level of inflammasome activation in monocytes vs. neutrophils remains to be investigated. With direct cellular staining of ASC and quantification of ASC levels in supernatant we were able to detect levels of speck formation and thereby verify the involvement of NLRP3 in the generation of IL-1β. Consistent with the findings by Stehlik et al.[49] we also show that ASC enhances IL-1β secretion in culture supernatant at low concentration observed for the monocytes at 4 and 8h while having a suppressing function at high concentration shown at 24h secreted in the supernatant.

Inflammatory ligands to TLRs often induce signaling events that result in changes of the expression of cell-characteristic surface markers and induction of different phenotype in blood cells and monocytes are not an exemption of this pattern.[33,50] It is acknowledged that the monocytes are composed of three cell subsets based on the expression of CD14 and CD16: classical $CD14^{+}CD16^{-}$, intermediate $CD14^{+}CD16^{+}$, and non-classical $CD14^{dim}CD16^{+}$ monocytes.[33] Each of these subsets possesses distinct biological functions and different responses to inflammation.[31,32] Flow cytometry assessment of monocytic populations presented in our study confirmed the existence and distribution of all three monocytic subsets are in line with previously reports.[51] Short (4h) incubations do not appear to induce major loss or gain of expression of monocyte markers CD14 and CD16, irrespectively of experimental condition. Similar observation with LPS stimulation for 4h was reported by others.[52] However, prolonged exposure to experimental conditions up to 24h induced a drop in the number of classical $CD14^{+}CD16^{-}$ monocytes and an increase of intermediate $CD14^{+}CD16^{+}$ cells. This re-distribution of the monocytic subsets suggest that the classical monocytes shift their phenotype towards the intermediate phenotype, because it is rather unlikely that the reduction in the number of classical and the increase of intermediate monocytes are events related to the cell death of the former and proliferation of the latter subset. The reduction of IL-1β at 24h coincidences with the phenotypical shift observed at the same time-point implying that this cytokine might be involved in the appearance of higher number of intermediate $CD16^{+}$ monocytes, since it has been reported that IL-1β induces CD16 expression in macrophages.[53] This intermediate subset is acquiring a more phagocytic phenotype due to the expression of CD16[54] and has a different cytokine profile compared to classical and non-classical monocytes.[31,55] Differences between monocytic cells were also observed with respect to the synthesis of IL-1β in this study. The pattern of IL-1β intracellular staining was very similar in tested donors and revealed that the classical monocytes are the predominant subset that synthesize most of IL-1β. Intermediate monocytes also contribute to the total IL-1β while the non-classical monocytes produce less IL-1β compared to the other monocyte subsets. Consistent with previous reports, we observed that the IL-1β production by monocyte subsets is LPS induced and augmented by ATP.[56,57]

To show that caspases are activated and necessary for IL-1β and IL-18 cleavage in our whole blood model, we performed experiments using AC-YVAD-CMK and AC-LEVD-CHO. Unfortunately no specific caspase inhibitors are available, AC-YVAD-CMK preferentially inhibits caspase-1 but also inhibits caspases 4 and 5,[58] whereas AC-LEVD-CHO preferentially inhibits caspase-4, but also caspases 1 and 5.[34] Both inhibitors inhibited IL-1β after LPS stimulation to a similar extent, however AC-YVAD-CMK inhibits IL-1β and IL-18 release more strongly after LPS+ATP stimulation (Fig 6). Another interesting finding is that IL-1β is released after stimulation with LPS alone (Fig 1), whereas IL-18 needs a secondary ATP trigger to be released (S2 Fig).

Recently it was shown that LPS activates the non-canonical pathway acting via caspase-4/5 without the need of a secondary trigger.[59] Vigano et al. show that the internalization of LPS via CD14/TLR4 activates a one-step inflammasome activation via syk and a Ca_2^+ flux, that results in the processing and release of IL-1β via caspase-4/5.[42] The involvement of the non-canonical inflammasome pathway explains both why IL-1β is released after LPS stimulation as well as why AC-LEVD-CHO is a less potent IL-1β and IL-18 inhibitor after LPS+ATP stimulation.

Several studies have shown benefits of stimulation of whole blood culture over purified cells for *in vitro* analysis of immune cell function including inflammasome response.[60,61]

In the present study, we show that the *ex vivo* whole blood stimulation is a simple and robust model for inflammasome activation, with detection of NLRP3 inflammasome signaling, caspase activation followed by IL-1β expression and secretion in human whole blood with minimal manipulation. This assay enables timely identification of possible overt inflammasome activation by novel therapeutics in human subjects prior application in first-in-human studies. Furthermore, this test by mimicking the physiological milieu to great extend is a reliable platform to study novel drugs that aim to suppress the inflammasome activation in pathological conditions characterized by profound production of IL-1β.

Fig 1 Inflammasome activation in whole blood cultures. (A) IL-1β dynamics and kinetics after inflammasome activation with LPS or LPS+ATP in six donors. **P<0.008 analyzed by two-way ANOVA with Tukey's multiple comparison test. (B, C) Cellular viability upon inflammasome activation in whole blood cultures. Cell percentage change over the course of 24h stimulation period compared to baseline (CTRL 0h) of monocytes (B) or neutrophils (C). (D, E) Cell viability stained with Annexin V/PI showing percentage of live cells for monocytes (D) and neutrophils (E). no significant differences between CTRL and LPS or LPS+ATP analyzed by mixed effects analysis with Tukey's multiple comparison test. Data are expressed as mean value ± standard error of the mean (SEM) of each stimulation condition, n=6; striped line represents baseline values at CTRL 0h.

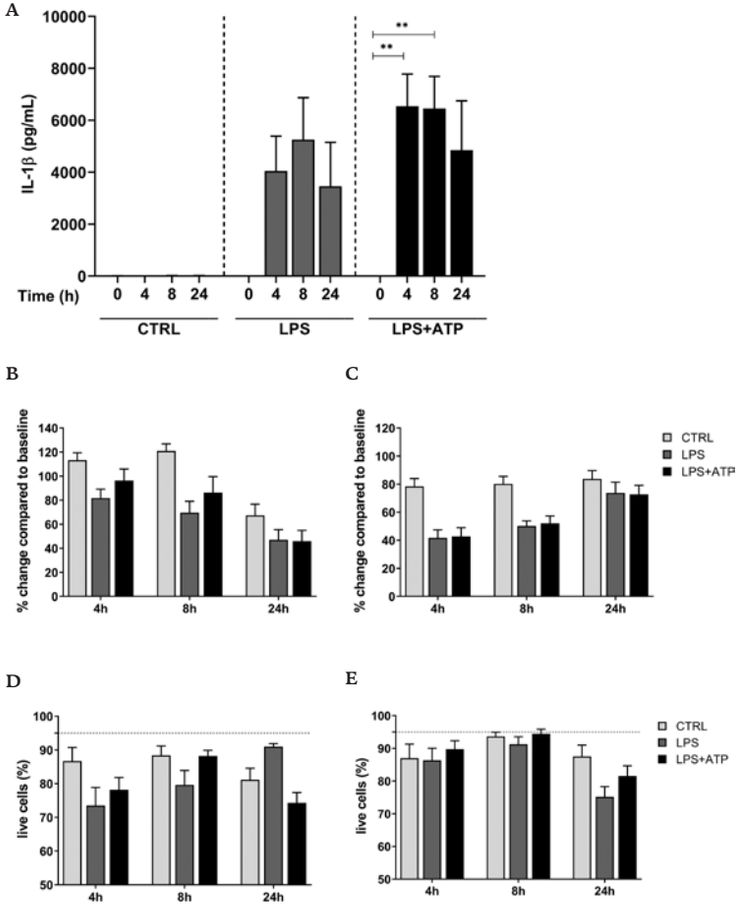


Fig 2 IL-1β expression in monocytes and neutrophils in whole blood. Mean fluorescence intensity (MFI) value ± SEM of IL-1β expression for each stimulation conditions (CTRL: open bars, LPS: grey bars, LPS+ATP: black bars) after 4, 8 and 24h in (A) $CD14^+$ monocytes, (B) $CD15^+CD16^+$ neutrophils. n=6. *P<0.05 and **P<0.008 analyzed by mixed effects analysis with Tukey's multiple comparison test.

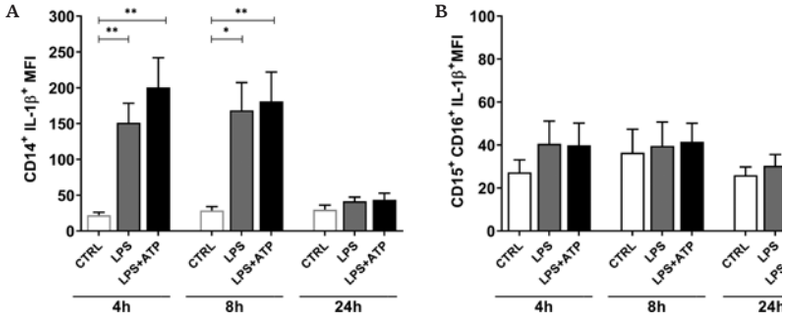


Fig 3 ASC detection in whole blood. Percentage ASC expression for each stimulation conditions (CTRL: open bars, LPS: grey bars, LPS+ATP: black bars) after 4, 8 and 24h in $CD14^+$ monocytic population (A) and $CD15^+CD16^+$ neutrophil population (B). (C) ASC/PYCARD detection in plasma upon inflammasome activation with LPS or LPS+ATP. Data are expressed as mean value ± SEM of each stimulation condition, n=2 (A,B) and n=6 (C).

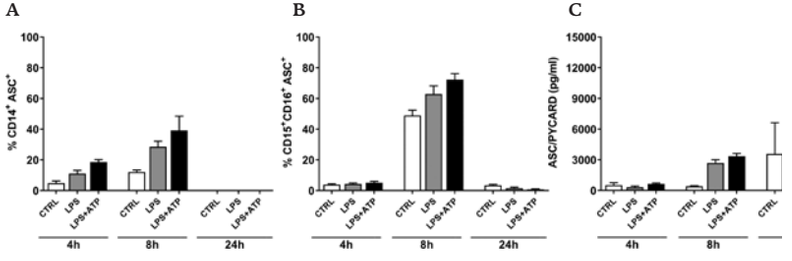


Fig 4 Monocytes phenotypically change during stimulation over time. Percentage expression of $CD14^+CD16^-$ monocytes (A), $CD14^+CD16^+$ monocytes (B) and $CD14^{dim}CD16^+$ monocytes (C). Data are expressed as mean value ± SEM of each stimulation condition, n=6. **P<0.005 and ***P<0.0001 analyzed by mixed effects analysis with Dunnett's multiple comparison test.

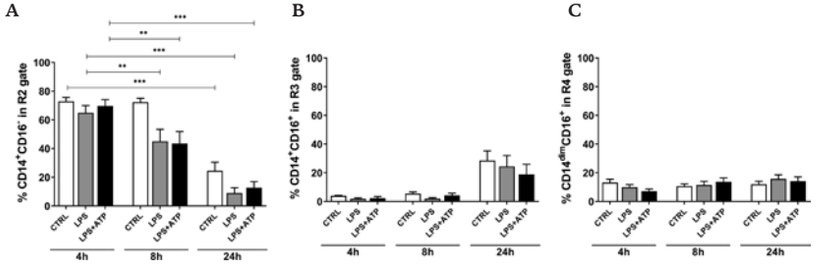


Fig 5 Caspase activation in whole blood assay. FAM-YVAD-FMK (FLICA) binding to active caspase-1 in monocytes after 1h (A) and 3h stimulation (B). Data are expressed as % of $FLICA^+$ cells in whole blood, n=4 (A) and n=3 (B).

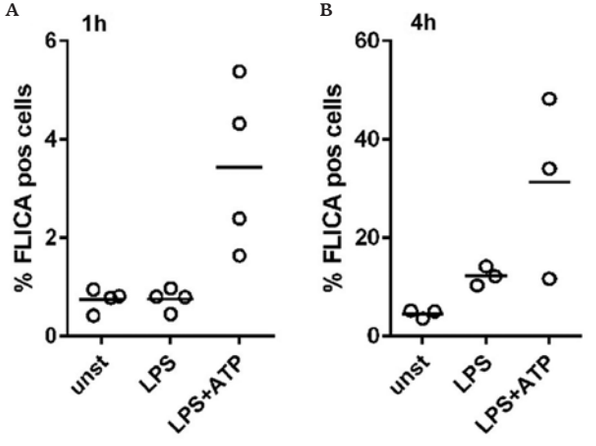
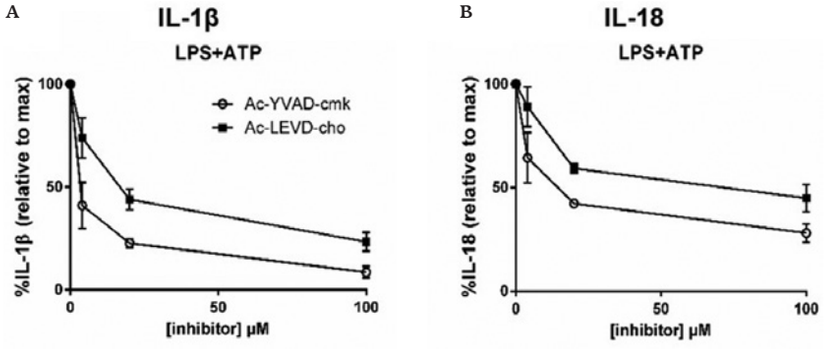


Fig 6 The effect of caspase-1 and caspase-4 inhibition on IL-1β and IL-18 secretion. Caspase inhibition of IL-1β release upon LPS+ATP (A) stimulation. Caspase inhibition of IL-18 release upon LPS+ATP stimulation (B). Data are expressed as mean ± % CV of duplicates from three independent experiments.



All mentioned supplementary figures and tables in this chapter can be found on the corresponding website by scanning this QR code.

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CHAPTER 5

Sample aging profoundly reduces monocyte responses in human whole blood cultures

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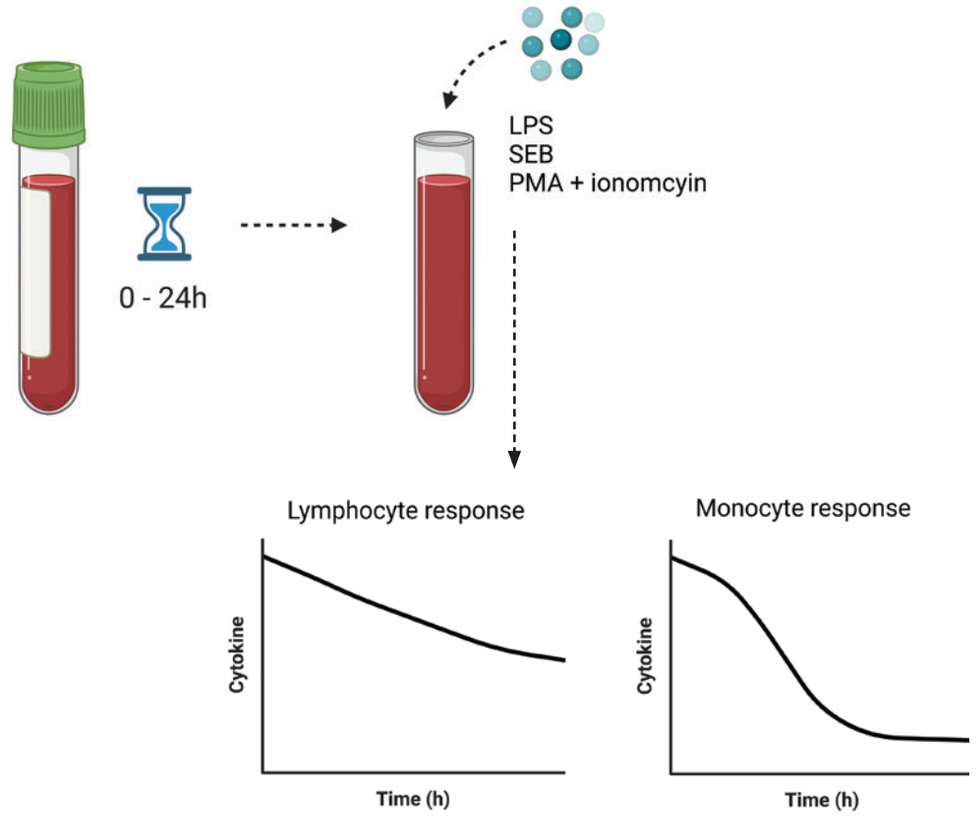
Graphical abstract



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Abstract

Human whole blood cultures are widely used for the investigation of physiological pathways and drug effects *in vitro*. Detailed information on the effect of "sample aging" (the time span between blood collection and experimental start) on the experimental outcome is not readily available in the public domain. We studied the effect of sample aging on the ability of immune cells to respond to cell-specific immune triggers (LPS, PMA/ionomycin, and SEB). Sample aging at room temperature profoundly inhibited the LPS-induced monocytic cytokine release in minimally diluted whole blood cultures. The reduction ranged from 20–50% after 30 minutes to 80–100% after 10 hours and differed between cytokines (IL-1β, IL-2, IL-6, IFNγ, and TNFα). Sample storage at 4°C or 37 ° C even worsened this. PMA/ionomycin- and SEB-induced cytokine release, both mainly T cell-driven, were also reduced by sample aging but to a lesser extent (20–50% after 24 hours). Intracellular cytokine staining revealed that the number of LPS-responding cells was not impacted by sample aging and reduced LPS responsivity could also not be explained by apoptosis or downregulated TLR4 expression. Thus, we speculate that sample aging induces an inhibitory pathway downstream from TLR4 in monocytes. These results underline the importance of quick sample handling when investigating innate immune responses in whole blood, especially for monocyte responses.

Introduction

Primary human immune cells are widely used for the investigation of physiological pathways and drug effects *in vitro*. Primary cells are often used for research on chronic diseases and for testing new therapies for a wide range of diseases. Such experiments are frequently performed on isolated leukocytes, mostly peripheral blood mononuclear cells (PBMCs). Cryopreserved PBMCs can be analyzed batchwise, so that samples collected at multiple clinical sites can be analyzed at one central laboratory. Alternatively, *in vitro* experiments can be conducted on whole blood samples, with the obvious benefit that this better resembles *in vivo* conditions.[1] Sample handling is minimal for *in vitro* experiments on whole blood samples: the only variable to control is the time span between blood collection and the start of the experiment. Although most publicly available literature on whole blood-based experiments does not specify this time span, it is evident that the "sample age" may vary substantially between and within experiments, depending on the clinical unit, the laboratory, and the donor population involved. Limited information is available on the effect of the sample age on the outcome of whole blood-based experiments. This is remarkable since a direct relationship between sample age and cell functionality (i.e., cell viability and cell responsiveness to immune triggers) could be expected. In PBMC cultures, apoptosis occurs spontaneously after prolonged sample storage.[2,3] In whole blood samples, blood settling causes red blood cell and platelet aggregation and alters cell function.[4] Another potential problem in whole blood cultures is the short lifespan of neutrophils.[5] These cells survive for less than 24 hours in the bloodstream and are prone to undergo apoptosis under suboptimal environmental conditions. Apoptotic neutrophils may have secondary effects on other leukocyte subsets in a whole blood based experiment. To overcome these problems, efforts have been made to preserve whole blood samples for longer periods of time. For example, cryopreservation of the whole blood has been shown to be feasible for experiments assessing Epstein-Barr virus (EBV) transformation, lymphocyte proliferation, and DNA extraction.[6–8] Freezing of fixed whole blood may also be appropriate for immunophenotyping.[9] However, granulocytes do not stay viable during the freezing process impacting the responses of other cell types.[10,11] The addition of phytohaemagglutinin (PHA) during sample storage avoided the apoptosis of lymphocytes,[2] and the addition of the polymer Ficoll to the whole blood prevented settling of red blood cells, limiting interference of aggregating red blood cells.[4] However, in addition, such chemicals may induce undesired cellular activation: PHA, for example, is known to stimulate T-cell proliferation.[12] An experimental setup without the addition of cell preserving chemicals is preferred when studying the natural behavior and effect of blood cells.

A limited number of studies describe the effects of whole blood storage on cellular responses. Unfortunately, these studies only focus on the effects on a specific cell population or cellular response, with contradictory results. The temperature at which the blood is stored may affect the functionality of monocytes, with low temperature storage (4°C for 24 hours) preferred over room temperature.[13] Another study demonstrated that when stored at room temperature, the number of cytokine-producing monocytes remains relatively stable, whereas interferon (IFN)γ- and IL-2-producing T cells declined during storage.[14]

We aimed to provide a comprehensive overview of the effect of sample aging on cell viability and stress and cellular reactivity to exogenous immune triggers. We investigated cell responsiveness by quantification of secreted cytokines, and we looked at the percentage of responsive cells for particular cell subsets (T-cells and monocytes) by means of the flow cytometric detection of intracellular cytokines. To obtain insight into cell-specific or pathway-specific effects of sample aging, we used immune triggers activating different immune cell subsets. T-cells were stimulated by simultaneous incubation with phorbol 12-myristate 13-acetate (PMA) and ionomycin and by staphylococcal enterotoxin B (SEB). PMA plus ionomycin induces a general T-cell activation via protein kinase C (PKC) and nuclear factor of activated T-cell (NFAT) signaling. Superantigen SEB activates up to 20% of all T-cells via the T-cell receptor β chain.[15] Monocyte activation was induced by lipopolysaccharide (LPS), a Toll-like receptor (TLR) 4 ligand.

Materials and methods

BLOOD COLLECTION

Blood was collected from healthy volunteers by venipuncture into sodium heparin-coated vacutainers or cell preparation tubes (CPT) containing sodium heparin (Becton Dickinson, NJ, USA) after written informed consent was obtained in accordance with Good Clinical Practice guidelines and the Declaration of Helsinki.

WHOLE BLOOD INCUBATIONS

Whole blood was simulated for 3 or 24 hours with LPS (2 ng/mL), SEB (100 ng/mL), or PMA/ionomycin (150 ng/mL and 7.5 µg/mL, resp.). All reagents were obtained from Sigma-Aldrich (Deisenhofen, Germany). For intracellular staining, brefeldin A (Thermo Fisher Scientific) was added to the cultures. Cultures were incubated at 37°C and 5% CO_2.

PBMC ISOLATION AND INCUBATIONS

PBMCs were collected from CPT samples and washed twice with PBS. PBMCs were counted using the MACSQuant 10 analyzer and resuspended at 1×10^6 cells/mL in RPMI1640 supplemented with 10% FBS. PBMCs were stimulated with 2 ng/mL LPS for 24 hours.

MITOCHONDRIAL FUNCTION

Mitochondrial membrane potential (MMP) was assessed in whole blood cultures after red blood cell lysis with RBC lysis buffer. Leukocytes were incubated with 0.5 µM JC-1 (Mitoprobe kit, Thermo Fisher Scientific) for 15 minutes. CCCP (10 µM) was used as a positive control. MMP was assessed in monocytes and T-cells with a MACSQuant 10 analyzer (Miltenyi Biotec). Mitochondrial function was expressed as the mitochondrial membrane potential, calculated as follows:[16]

$$\Delta\psi m = \frac{(FL1 : FL2)}{(Fl1_{CCCP} : FL2_{CCCP})} * 100\%$$

CYTOKINE MEASUREMENTS

IFNγ, TNFα, IL-1β, and IL-6 from whole blood culture supernatants were measured using the V-plex inflammatory panel-I kit from Meso Scale Discovery (Rockville, MD, USA). IL-2 and IL-10 were measured by ELISA (Thermo Fisher Scientific).

Results

SAMPLE AGING RESULTS IN A RAPID DECLINE OF LPS-DRIVEN RESPONSES, WHEREAS PMA- AND SEB-DRIVEN ARE LESS AFFECTED

Whole blood was collected and stored at room temperature or at 4°C until incubation experiments were started (immediately after blood collection and 0.5, 1, 2, 4, and 10 hours after blood collection). Whole blood cultures were stimulated with LPS, SEB, and PMA/ionomycin. Sample aging strongly reduced LPS-induced IFNγ, IL-1β, IL-6, and TNFα release (Figure 1A). A delay in the start of the incubation of only 0.5 hour already resulted in a loss of 30–50% of the cytokine profound (Figure 1B). After 0.5 hour, hardly any effect of sample aging was observed. Maximal aging of 10 hours resulted in a loss of 25–60% of the cytokine response (IFNγ, IL-6, and TNFα). In contrast to LPS responses, sample storage at 4°C better preserved PMA/ionomycin induced cytokine release compared to storage at room temperature. SEB responses were highly variable between subjects, but no strong indications were found for a time-dependent reduction in cell responses (Figure 1C). Cytokine release in unstimulated samples remained low (<50 pg/mL) for all sample ages investigated (data not shown). Sample aging did not induce the release of the anti-inflammatory cytokine IL-10 (all responses < 25 pg/mL, data not shown). Given the large temperature-dependent effect on LPS-driven responses, sample storages at 37°C and at room temperature were compared in a separate experiment (Figure 1D). The whole blood was stimulated with LPS immediately after blood collection and after 2, 10, and 24 hours of storage. Sample storage at 37°C resulted in an even more rapid decrease in IL-6 and TNFα production compared to room temperature.

SAMPLE AGING DOES NOT AFFECT CELL VIABILITY, MITOCHONDRIAL FUNCTION, OR TLR4 EXPRESSION

Since LPS stimulation results in a mainly monocyte-driven response, and SEB and PMA/ionomycin are mainly T-cell stimuli, we hypothesized that monocytes are more prone to undergo cell death due to sample aging. However, absolute counts of $CD3^+$ T-cells, $CD14^+$ monocytes and granulocytes (as gated in FSC/SSC scatter plot) did not decrease with prolonged sample storage (Figure 2A). Also no increase in number of dead cells was observed, as measured by propidium iodide staining (data not shown). To check whether sample aging rendered the cells apoptotic, annexin V stainings were performed (Figure 2B). For all tested conditions, the percentage of annexin V positive cells remained below 5%, demonstrating that neither monocytes nor T-cells are in a more apoptotic state at the beginning of the culture experiments after prolonged sample aging. Next, the effect of sample aging on cell functionality was investigated by measurement of the mitochondrial membrane potential (MMP), a marker for cellular stress (Figure 2C). Also based on MMP, no effect of sample aging was detected that

could explain the significant reductions in LPS-induced cytokine responses. Finally, it was investigated whether the impaired LPS responses in aged blood samples could be explained by a reduced recognition of the trigger. However, sample aging did not significantly affect TLR4 expression on monocytes (Figure 2D).

SAMPLE AGING DOES NOT AFFECT THE NUMBER OF RESPONDING CELLS

To explore whether the impaired LPS response after sample aging could be explained by a reduction in responding number of cells, additional experiments were conducted, but now with intracellular cytokine production as endpoint. Whole blood cultures were started directly after blood collection, and with a delay of 1 and 4 hours and storage at room temperature. After LPS stimulation, the percentage of cytokine-producing monocytes was high: approximately 80% produced IL-6, and 95% produced TNFα (Figure 3A). T-cells and monocytes remained negative for IFNγ staining after LPS stimulation (data not shown).In response to PMA/ionomycin, approximately 45% of the T-cells produced IL-2 and IFNγ, 30% produced IL-6, and 65% produced TNFα (Figure 3B). Monocytes responded to PMA/ionomycin as well: approximately 10% produced IL-6, and 20% produced TNFα. Importantly, sample aging did not affect the number of monocytes or T-cells responding to LPS or PMA/ionomycin (Figure 3A and 3B).

THE ADDITION OF CULTURE MEDIUM DOES NOT PRESERVE THE WHOLE BLOOD RESPONSE

To assess whether the addition of culture medium prevents the sample aging-dependent loss of cellular responsiveness to LPS, RPMI1640 was added to the whole blood samples directly after blood collection (Figure 4). The addition of RPMI to the blood cultures did not result in preservation of cell responsiveness: LPS-induced cytokine responses were affected to the same extend by sample aging in the absence and presence of RPMI. To test whether the sample aging effect could relate to influences of dying granulocytes or granulocyte products, an experiment was performed with PBMCs. Cells were stimulated with 2ng/ml LPS directly after PBMC isolation (t=0h) or after up to 6 hours of aging (Figure 5). The sample aging effect on LPS responsiveness was comparable between the PBMC experiment (Figure 5) and earlier whole blood experiments (Figures 1 and 4) concerning IL-6 (mild reduction in cytokine release) and IL-1β (significant reduction in cytokine release). Interestingly, the effect on LPS-induced TNFα release was opposite between both setting: whereas sample aging reduced TNFα release in whole blood (Figures 1 and 4), it strongly enhanced TNFα release in PBMCs (Figure 5).

Discussion and conclusions

Human whole blood cultures are widely used for investigation of physiological pathways and drug effects *in vitro*. Detailed information on the effect of 'sample aging' (the time span between blood collection and experimental start) on the experimental outcome is not readily available in the public domain. This is an important knowledge gap, especially since whole blood-based pharmacodynamic assays have become increasingly important for the guidance of first-in-human clinical pharmacology studies with investigational medicinal products. Whole blood challenges are commonly applied to study the effect of immunomodulatory compounds on innate immune responses, such as *ex vivo* LPS and PHA stimulations.[17-19] No strict protocols or criteria apply for such whole blood cultures, and a main factor that potentially confounds experimental outcome is the 'sample age', the time span between blood collection and start of the experiment. To provide more insight into the effect of sample aging on innate immune responses induced *ex vivo*, we stimulated whole blood samples with cell-specific immune triggers (LPS, PMA/ionomycin, SEB). We studied the effect of sample aging on the ability of immune cells to respond to these triggers. Whole blood was minimally diluted (9% dilution) to resemble *in vivo* conditions as close as possible.

Sample aging at room temperature, but also at 4°C or 37°C, profoundly inhibited LPS-induced cytokine release. At room temperature, the reduction ranged from 20-50% after 30 minutes to 80-100% after 10 hours, with the strongest reductions observed for IFNγand the smallest reduction for IL-6. An LPS-driven cytokine response in whole blood samples is mainly monocyte-derived.[20] PMA/ionomycin- and SEB-induced cytokine release, both mainly T-cell-driven, were also reduced by sample aging but to a lesser extent (20-50% after 24 hours). Apparently, in a whole blood setting monocytes are more affected by sample aging than T-cells. PMA/ionomycin stimulation of whole blood drives a T-cell response, but also directly activates monocytes. For example, monocytes produce IL-2 upon PMA/ionomycin stimulation.[21] We observed IL-6 and TNFα production by

monocytes after PMA/ionomycin stimulation. Therefore, the slight reduction in IL-6 and TNFα in aged whole blood samples stimulated with PMA/ionomycin is probably explained by a lower responsiveness of the monocyte fraction, and not of the T-cell fraction.

Previous reports on the effect of sample aging show that monocyte responses do not diminish after prolonged sample storage.[13,14] Schultz et al. concluded that prolonged sample storage resulted in a decreased lymphocyte response, without affecting monocyte responsiveness.[14] However, these conclusions were based on intracellular cytokine staining only.In our experiments, intracellular cytokine staining also showed that the number of LPS-responding cells was not impacted by sample aging, so the reduced responsiveness to LPS could not be explained by fewer cells responding to the trigger. In previous experiments, whole blood cultures were diluted with culture medium providing nutrients for the cells,[14,22] which may preserve cell responsiveness during prolonged periods of sample storage. Therefore, we repeated our experiment with RPMI1640 culture medium added to the aging samples, but this did not prevent or reduce sample aging-dependent decreases in cytokine production.

Since monocytes can die via an apoptotic process in the absence of specific activation stimuli,[23,24] we investigated whether reduced LPS responsiveness could be explained by cell death or cellular stress. However, we did not find any indication for a sample aging-dependent reduction in number of viable immune cells, elevated apoptotic cells, or altered mitochondrial membrane potential. This is in line with literature reporting that apoptosis in whole blood cultures only develops after at least 24 hours of sample storage.[3] Since uptake of apoptotic cell fragments may suppress the cytokine production by macrophages,[25] the conclusion that reduced cytokine responses in our experiments do not coincide with cell death or cellular stress is informative, and suggests that other physiological mechanisms may explain the observed effects of sample aging. We also demonstrated that reduced cytokine release in aging samples did not correlate with downregulated TLR4 expression, or with an enhanced IL-10 production (data not shown). Thus, we speculate that sample aging induces an inhibitory pathway downstream from TLR4 in monocytes. Alternatively, changes in the expression of cytokine receptors on monocytes may have caused autocrine consumption of cytokines, but this was not investigated.

All the cytokine release experiments described in this manuscript were performed with incubation durations of 24 hours. This time span is sufficiently long to not only allow primary LPS-driven responses, but also secondary leukocyte responses initiated by LPS-induced factors, or even by factors induced by sample aging. One potentially relevant factor driving secondary responses are granulocyte-related products. For example, granulocyte contamination in PBMC cultures reduced T-cell responses to PHA and FMLP.[26] Granulocytes are known to have a short lifespan and to be sensitive to sample handling. Spontaneous activation of granulocytes occurs after 6-8 hours after venipuncture.[26] In our experiments, no apoptosis of granulocytes was observed in the first 10 hours after sample collection (data not shown). Since we did not assess the level of granulocyte activation, it is theoretically possible that this may have had an effect on the immune responses in our whole blood cultures. However, we showed that sample aging-dependent alterations in some LPS-induced cytokine responses were not only observed in whole blood, but also in PBMC cultures, suggesting that granulocyte-derived factors alone do not explain the observed effects of sample aging.

Significant levels of IFNγ were released upon LPS, SEB and PMA/ionomycin stimulation in whole blood cultures. However, intracellular staining showed that LPS stimulation did not induce IFNγ production in either monocytes or T-cells. There are different potential explanations for this observation. Possibly, a different cell type accounted for the IFNγ production after LPS stimulation, for example NK cells and B cells are known to produce IFNγ upon induction of innate immune responses.[27] Alternatively, LPS-induced IFNγ release may have been secondary to a primary LPS-driven effect, and the incubation time for the intracellular cytokine experiments was too short to allow such a secondary response. Further investigation into this observation falls beyond the scope of this paper.

These results underline the importance of the use of fresh samples when investigating innate immune responses in whole blood. Given the ever increasing application of whole blood challenge tests as pharmacodynamic readout measure in early phase clinical pharmacology trials, a better understanding of the conditions affecting the outcome of such tests is critical. We demonstrated that sample aging primarily affects monocyte responses, and that this cannot be explained at the level of cell viability or ligand recognition.

Figure 1 Cytokine release in whole blood culture supernatants (as % of the response at t=0, average plus SD). Whole blood of 3 donors was stimulated for 24 hours with LPS (A), PMA/ionomycin (B) or SEB (C). Whole blood of 5 donors was stimulated for 24 hours with LPS (D). The x-axis indicates the sample age (time span between blood collection and start incubation). Blood was stored at room temperature (continuous line), 4°C (dashed line panel A/B/C), or 37°C (dashed line panel D).

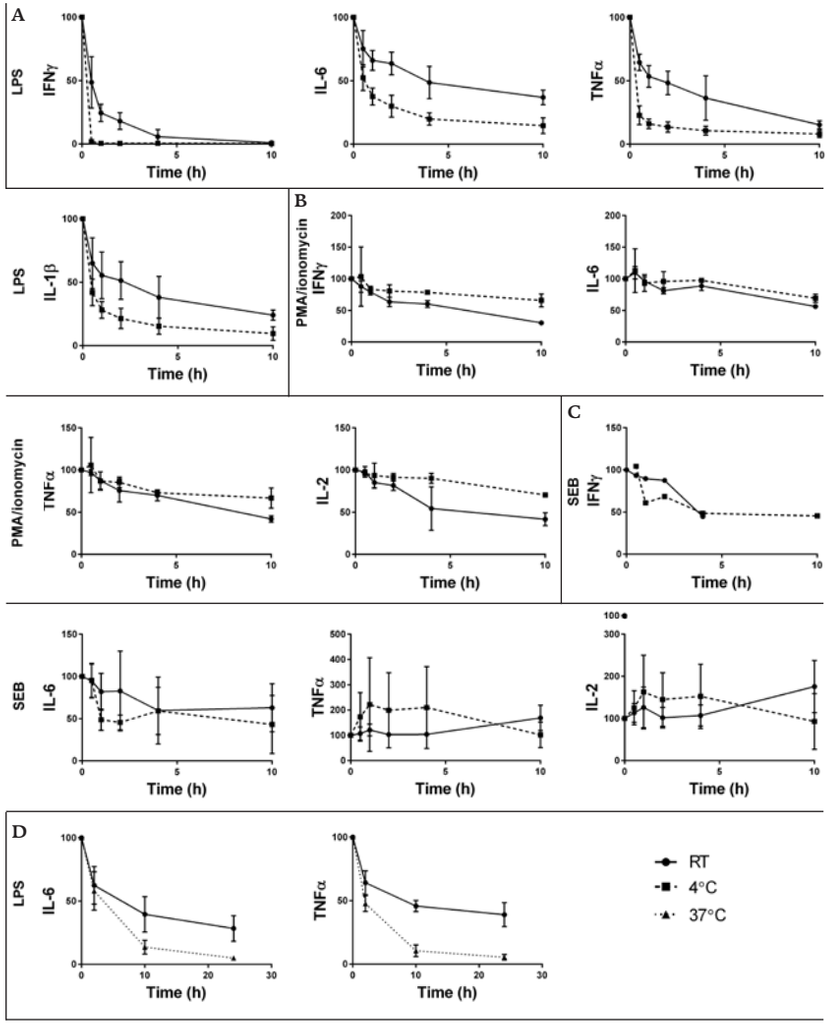


Figure 2 Cell viability (cell counts, annexin V, MMP) and TLR4 expression in whole blood cultures (average plus SD). In whole blood cultures of 6 donors, absolute cell counts (T-cell, monocyte and granulocytenumbers; (A), apoptosis (annexin V-positive monocytes; (B), mitochondrial function (MMP for T-cells and monocytes; (C) and TLR4 expression (TLR4-positive monocytes; (D) were quantified. The x-axis indicates the sample age (time span between blood collection and start incubation). Blood was stored at room temperature (continuous lines) or 4°C (dashed lines).

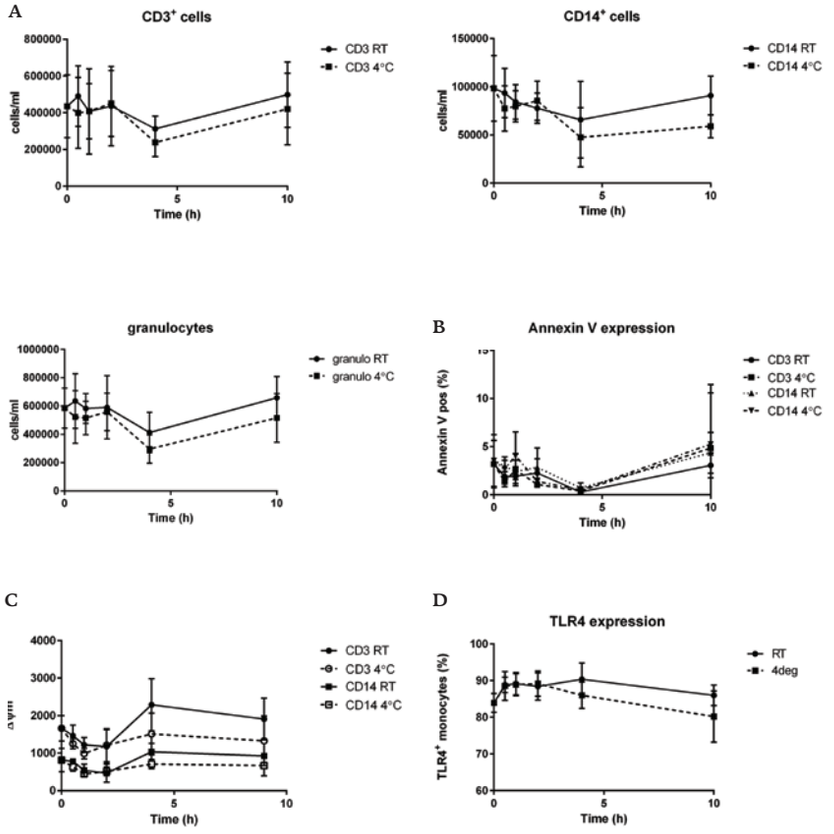


Figure 3 Cytokine-producing cells in whole blood cultures (average plus SD). Whole blood of 5 donors was stimulated for 3 hours with LPS (A) or PMA/ionomycin (B) in the presence of brefeldin A to block cytokine secretion. The percentage positive cells for intracellular cytokines was quantified for T-cells (continuous lines) and monocytes (dashed lines). The x-axis indicates the sample age (time span between blood collection and start incubation). Blood was stored at room temperature. T-cells and monocytes remained negative for IFNγ staining after LPS stimulation.

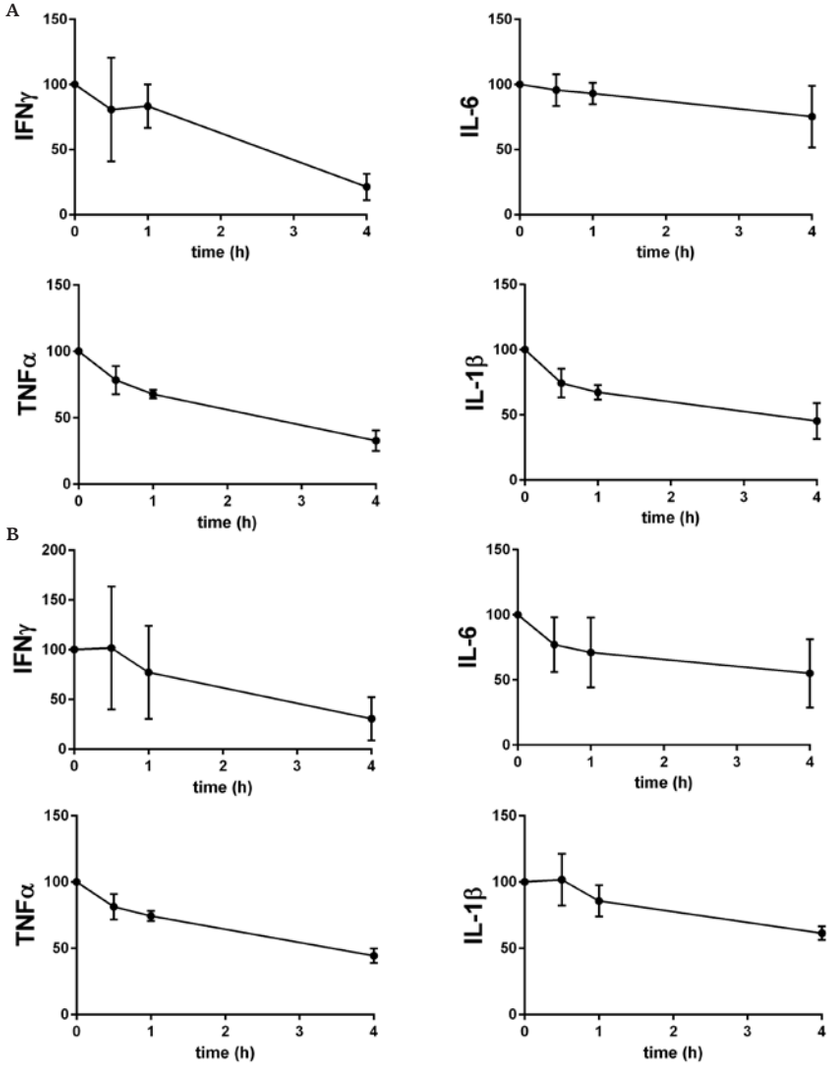


Figure 4 Cytokine release in whole blood culture supernatants; RPMI effect (as % of the response at t=0, average plus SD). Whole blood of 3 donors was stimulated for 24 hours with LPS in the absence of RPMI (A), or diluted 1:1 with RPMI during aging (B). The x-axis indicates the sample age (time span between blood collection and start incubation). Blood was stored at room temperature.

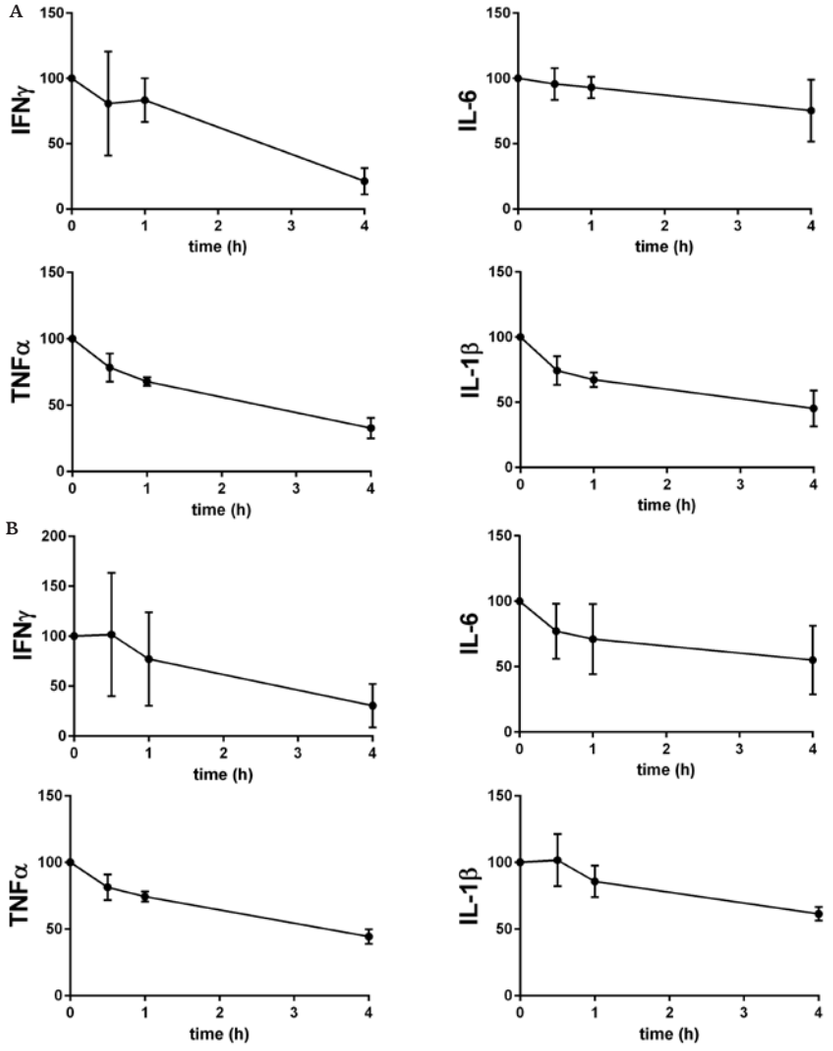
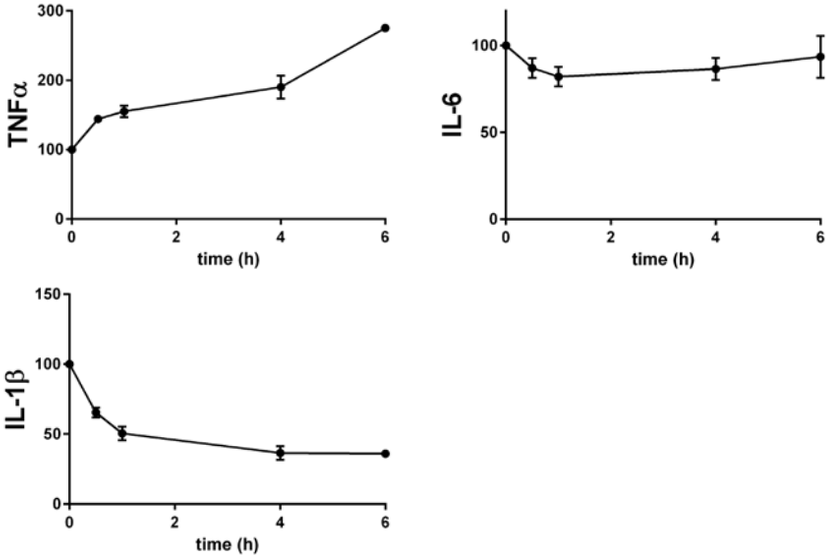


Figure 5 Cytokine release in PBMC culture supernatants (as % of the response at t=0, average plus SD). Isolated PBMCs of 2 donors were stimulated for 24 hours with 2 ng/ml LPS directly after isolation or after aging up to 6 hours in RPMI + 10% FBS. The x-axis indicates the sample age (time span between PBMC isolation and start incubation). Cells were stored at room temperature. For IFNγ most samples remained below LLOQ.



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SECTION II

IMMUNOMODULATION OF ATHEROSCLEROSIS

CHAPTER 6

The effect of a 13-valent conjugate pneumococcal vaccine on circulating antibodies against oxidized LDL and phosphorylcholine in man, a randomized placebo-controlled clinical trial

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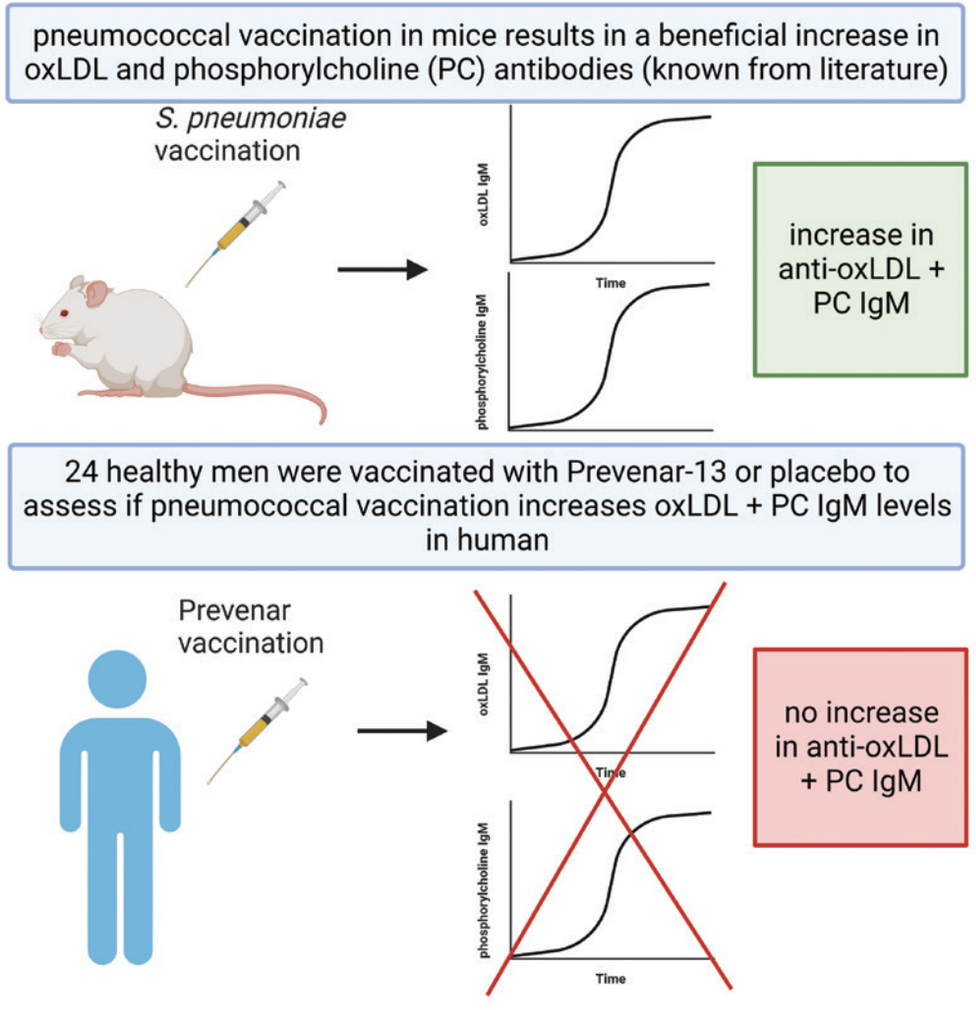
Graphical abstract



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Abstract

In mice vaccination with *Streptococcus pneumoniae* results in an increase in anti-OXLDL IgM antibodies due to mimicry of anti-phosphorylcholine (present in the cell wall of *S. pneumoniae*) and anti-OXLDL IgM. In this study we investigated the human translation of this molecular mimicry by vaccination against *S. pneumoniae* using the Prevenar-13 vaccine. Twenty-four healthy male volunteers were vaccinated with Prevenar-13, either 3 times, 2 times or once in a double blind, placebo controlled, randomized single center clinical study. Anti-pneumococal wall, OXLDL and phosphorylcholine antibody levels were measured at a fixed serum dilution, as well as circulating lipid levels in the course of 68 weeks. A significant increase in anti-OXLDL IgG and IgM is seen in the group receiving two doses 6 months apart compared to placebo. However, these differences were not observed in the groups receiving a single dose, two dosages 1 month apart, or three dosages. This study shows that vaccination with Prevenar-13 does not result in robust anti-OXLDL IgM levels in humans. Further research would be required to test alternative pneumococcal-based vaccines, vaccination regimens or study populations, such as cardiovascular disease patients.

Introduction

Oxidized LDL (OXLDL) particles play a key role in the etiology of atherosclerosis.[1] In the vessel wall, OXLDL is recognized and phagocytosed by macrophages primarily via scavenger receptors leading to foam cell formation.[2] Macrophage foam cells are hall mark cells of atherosclerotic lesions and participate in the inflammatory responses that mediate smooth muscle cell migration and proliferation and extracellular matrix production, and thereby stimulate atherosclerotic plaque progression.

Several mouse studies showed that IgM antibodies against OXLDL are atheroprotective.[3,4] IgM antibodies against oxidized particles facilitate the clearance of apoptotic cells, thereby promoting the resolution of inflammation.[5,6] Additionally, these antibodies neutralize the proinflammatory effects of oxidized phospholipids.[7,8] Inhibition of scavenger receptor-mediated OXLDL uptake by macrophages prevents the formation of foam cells and subsequent progression of atherosclerotic plaque formation.[3,9] In clinical studies, OXLDL-specific IgM has been reported to be a protective factor for atherosclerosis development, correlating with cardiovascular disease incidence and clinical outcome.[10-13]

In contrast to IgM, the role of OXLDL-specific IgG in atherosclerosis is thought to be atherogenic. OXLDL-IgG complexes have been shown to induce survival of plaque-resident monocytes[14] and secretion of proatherogenic cytokines by mast cells.[15] Clinical studies showed a correlation between OXLDL-IgG antibodies and acute coronary syndrome, suggesting an untoward role of this antibody in plaque destabilization.[16] In human, OXLDL-specific IgG antibody titers correlated inversely to the OXLDL serum concentration[17] and, in mouse, serum cholesterol levels,[18] which suggests that OXLDL-specific IgG facilitates phagocytosis of OXLDL by macrophages.

Mouse experiments showed that certain IgM clones binding OXLDL bind phosphorylcholine (PC) of oxidized – but not unoxidized – phospholipids.[3,19] Importantly, Binder et al. showed in LDLR knockout mice that vaccination against *Streptococcus Pneumoniae* using pneumoccocal extracts induced high titers of OXLDL-specific IgM, subsequently leading to a decrease in atherosclerotic lesions.[19] This effect was explained by the fact that PC is present as part of the capsular polysaccharide of *S. Pneumoniae*. Moreover, immunization with PC conjugated to carrier proteins also induced OXLDL-IgM and decreased the extent of atherosclerosis in APOE knockout mice.[20,21]

Autoantibodies against PC are also found in man, where low levels of PC-IgM autoantibodies correlate with a higher incidence of cardiovascular disease.[22-25] Moreover, pneumococcal-specific IgG and OXLDL-specific antibody titers correlated significantly in subjects who had received pneumococcal vaccination,[26] although there are also reports of an absent effect of pneumococcal vaccination on OXLDL-specific IgM levels.[27]

The present proof-of-concept study investigated the human translation of the observed effects of pneumococcal immunization in mice. Healthy human volunteers were vaccinated with a 13-valent conjugated pneumococcal vaccine (Prevenar-13®), and the induction of PC- and OXLDL-specific antibodies was measured.

Materials and Methods

This was a double-blind, randomized, placebo controlled, parallel, single center study with twenty-four healthy males between 18 and 45 years of age. The study was performed at the Centre for Human Drug Research in Leiden, The Netherlands. Participants were recruited via advertisements and social media. Participants were assessed to be generally healthy based on a complete medical screening and had no previous exposure to the 13-valent pneumococcal vaccine. All participants gave written informed consent prior to any study-related activity. The study was approved by the Ethics Committee of the Leiden University Medical Centre (LUMC) and declaration of Helsinki principles were followed. The study is registered in the Dutch Trial Registry (Nederlands Trial Register, NTR) under study number NTR5643 and took place for all participants simultaneously between March 2016 and October 2017. This study was funded by the European Union, call FP7-HEALTH-2013-INNOVATION, project ID 603131.

VACCINATION SCHEDULE

The 13-valent conjugated pneumococcal vaccine (Prevenar-13®) used in this study was from a single batch (batch no. MU7958). The presence of residual PC in the vaccine preparation was confirmed by ELISA using the PC-specific MAB IgM E06. Placebo consisted of 0.9% NACL solution. Since there are clear visual differences between these vaccinations, three physicians were unblinded for administration of the vaccine. These physicians were not otherwise involved in the study. Vaccinations took place at 3 time points: at

baseline, at 4 weeks and at 28 weeks. Subjects were randomized in a consecutive order based on eligibility. The randomization code was generated using SAS v9.4 for Windows (SAS Institute Inc., Cary, NC, USA) by an independent statistician. The randomization code was only made available for data analysis after study completion. There were five different treatment arms, as displayed in figure 1. In the mouse study 3 immunizations were enough for OXLDL-specific IgM induction. In the mouse study proteinase treated *S. Pneumoniae* extracts were used. In this design, the power to detect differences between placebo and active treatment arms was optimized between baseline and at the 28 week time point (PP vs. AA vs. AP, n=8 per group).

ANTIBODY MEASUREMENTS

K_2EDTA plasma antibody levels to Prevenar, PC-BSA, and $CUSO_4$-oxidized LDL (OXLDL) were measured by chemiluminescent ELISA as reported previously.[28] In brief, Prevenar (Pfizer) was coated at 1:5000, PC-BSA (Biosearch Technologies) and OXLDL at 5 ug/ml in PBS/EDTA. IgM antibodies were measured at a dilution of 1:500 and IgG antbodies at 1:1000. Binding of IgG subclasses to Prevenar was measured at a dilution of 1:100 and to PC-BSA at 1:100 for IgG3 and IgG4, and 1:500 for IgG2. Serum levels of total cholesterol, low density lipoprotein (LDL), high density lipoprotein (HDL) and triglycerides were measured by the chemistry lab of the Leiden University Medical Center on the Cobas P800 analyzer (Hoffmann-La Roche, Basel, Switzerland).

POWER CALCULATION

In man, the median anti-OXLDL IgG levels in the healthy, unvaccinated population is around 50 U/l, with an interquartile range of around 25-75 U/l.[26] Anticipating an immune response minimally resulting in a 5-fold rise in IgG and IgM antibody levels, and based on an inter-subject variability of 50% in basal IgG and IgM levels,[19,24,26] a sample size of 4-8 subjects per group (dependent on the contrast) will be sufficient to meet the study objectives. This was a conservative approach considering in the magnitude of the OXLDL-specific IgM response observed in the murine model.[19]

STATISTICAL ANALYSIS

Data are presented as mean ± standard deviation (SD). In case of non-normal distribution, parameters were log-transformed. Repeatedly measured variables were analyzed with a mixed model analysis of variance with fixed factors treatment group, time and the interaction of treatment group and time as fixed factor and subject as random factor. Primary endpoints (prevenar-specific IG levels, OXLDL-specific IG levels and PC-specific IG levels) were compared between treatment groups for the 0-4 week window, the 4-28 week window, and the 28-68 week window. As a secondary endpoint, lipid levels in circulation were measured. Estimated differences were calculated between the groups. A positive value indicates a higher estimated value for the active group, a negative value indicates a lower value for the active group. The analysis was performed in SAS v9.4 (SAS Institute, Cary, NC, USA).

Results

Twenty-four healthy volunteers were included in the study, baseline characteristics can be found in table 1. One subject withdrew consent after two weeks for non-study related reasons (Figure 1). This subject was randomized to the active-active-placebo treatment arm and was not replaced.

ANTI-PREVENAR ANTIBODIES

Prevenar-specific IgG was significantly increased in all subjects who received any active treatment compared to placebo treated subjects, see table II and figure 2A. Prevenar-specific IgM was significantly increased in subject who received any active treatment up to 28 weeks, however after 68 weeks only subjects receiving three active doses had a significantly increased IgM level compared to placebo.

ANTI-OXLDL AND ANTI-PC ANTIBODIES

No difference was observed in PC-specific IgG levels compared to placebo, for any of the active treatment groups. Similarly, no difference was observed for PC-specific IgM levels between active treatment groups and placebo, with the exception of IgM levels being higher in the APA group compared to PPP during the study period(ED: 9409.7, 95% CI: 3227.5 - 15591.9, P=0.005) (figure 3). There were no differences in OXLDL-specific IgG and IgM antibodies between active and placebo treated subjects up to 28 weeks. However, at 68 weeks, subjects who received an active treatment at baseline and after 28 weeks (APA) had an increased OXLDL-specific IgG level

compared to subjects receiving 3 placebo injections (PPP) with an estimated difference (ED) of 9913 (95% CI: 3141 - 16686; P=0.007). As shown in figure 2B difference between these groups was also observed for OXLDL-specific IgM levels (ED: 12235, 95% CI: 4179 - 20290; P=0.005).

LIPIDS

The levels of total cholesterol (A), LDL (B), HDL (C) and triglycerides (D) of all groups during the study are depicted in Figure 3. No significant differences were found between treatment groups, with the exception of subjects receiving a single active treatment at the beginning of the study (APP) had a significant higher triglyceride level compared to placebo (PPP) (ED: 7.9%, 95%CI 18 % - 171%; P=0.009).

Discussion

The present study evaluated the effect of a 13-valent conjugate pneumococcal vaccine on the induction of anti-OXLDL and anti-PC antibodies and cholesterol levels in humans. Several vaccination regimens were tested, where subjects received either 1, 2 or 3 doses of Prevenar-13 over a period of 28 weeks compared to placebo. Despite the induction of an adequate anti-Prevenar 13 antibody response, there was no evident induction of either PC-specific or OXLDL-specific antibodies. Prevenar-13 immunization induced a significant IgG2 response when subject were immunized at least twice, while levels of IgG3 and IgG4 were not altered (Figure S1). In one active treatment group a statistically significant difference in PC-specific and OXLDL-specific antibody levels was observed compared to placebo. This occurred in the group receiving two doses of the vaccine at the start of the study and after 28 weeks (APA). A significant increase in PC-specific IgM and OXLDL-specific IgM and IgG was observed at 68 weeks. Interestingly, we observed a significant increase in PC-specific IgG3 for the group receiving two vaccinations 4 weeks apart (AAP) compared to placebo (PPP) (Figure S1 panel B). Elevated OXLDL-specific IgM is believed to be atheroprotective,[10-13] but the role of OXLDL-specific IgG levels is not fully elucidated. Laczik et al.[16] show that increased OXLDL-specific IgG levels correlate with acute coronary syndrome, while immunization with OXLDL, resulting in an increase in OXLDL-specific IgG, resulted in decreased plaque development in several mouse models.[18] Furthermore, OXLDL levels are inversely correlated with OXLDL-specific IgG serum levels.[17] Binder et al.[19]showed *S. Pneumoniae* immunization in mice induced a much stronger OXLDL-specific IgM response than an OXLDL-specific IgG response (100.000 vs 10.000 RLU/100ms). The current clinical data are at odds with this observation, though the observed anti-OXLDL responses in the APA group may indicate that the timing of vaccination could be important.

Previous studies have investigated the 23-valent polysaccharide pneumococcal vaccine as a means to elicit OXLDL-specific antibodies in man, with conflicting results.[26,27,29] One study reported that after vaccination with the 23-valent vaccine, an increased OXLDL-specific IgG antibody titer was observed compared to healthy, unvaccinated individuals (248 U/l vs. 55 U/l). An effect of vaccination on IgM was not reported. In the other two studies, no association between vaccination status and OXLDL-specific antibodies was observed. There are, however, key differences between these studies and the present study. First, patients in all three referenced studies only got a single vaccination, whereas in the present study, up to 3 vaccinations were given. Second, there is a major difference between the 23-valent polysaccharide vaccine that was used in these studies and the 13-valent polysaccharide conjugate vaccine that was used in the present study. The latter is constituted of cell wall polysaccharides that have been conjugated to a protein. The 13-valent vaccine is thereby considered to be more powerful in eliciting an antibody response against *Streptococcus pneumoniae*.[30,31] These data were the basis for the selection of the 13-valent vaccine for the current clinical study.

Although the sample size per group was relatively small in the current study (n=4 for treatment groups, n=8 for placebo), the study was sufficiently powered to detect Prevenar-13-induced rises in IgM titers, had these occurred as in the murine experiments.[19]

The induction of anti-pneumococcal wall saccharide antibodies demonstrates that the 13-valent vaccine was effective for its intended use. However, the vaccine did not elicit a robust OXLDL-specific IgM response, as observed in mouse experiments. One explanation for the poor induction of OXLDL-specific antibody responses by Prevenar 13 may be that the murine immune response is poorly translatable to humans. Human and mice have numerous discrepancies in their innate and adaptive immune systems, such as cytokine receptor and costimulatory molecule expression and function.[32,33] Moreover, murine studies are commonly performed

in inbred strains, with limited genetic variability between mice. This, and the fact that mice are kept in a more sterile environment, results in a smaller immune diversity compared to man.[34] On the other hand, the mild but significant induction of OXLDL-specific antibody responses in one active treatment group (APA) does support further clinical investigation of mimicry between pneumococcal vaccination and OXLDL.

In summary, vaccination of humans with Prevenar-13 did not significantly increase PC-specific antibodies and OXLDL-specific antibodies nor resulted in significant changes in plasma lipids. Nevertheless, subgroup analyses suggested an induction of PC-specific and OXLDL-specific IgM and IgG in individuals receiving two doses 6 months apart. Future research should investigate alternative pneumococcal vaccines (driving more significant anti-PC antibody responses), vaccination regimens, or study populations to confirm or refute the hypothesis that molecular mimicry underlying pneumococcal-driven anti-OXLDL responses as observed in mice occur in man.

Table 1 Baseline characteristics

	N=24
Age (years)	28.5 ±8.5
Gender male (%)	100
Ethnicity Caucasian (%)	100
Height (cm)	180.5 ±5.3
Weight (kg)	75.0 ±11.0
BMI (kg/m^2)	23.0 ±3.2
Heart rate (min^{-1})	58.5 ±9.0
Systolic blood pressure (mmHG)	123 ±9.3
Diastolic blood pressure (mmHG)	75.1 ±6.7

Table 2 Estimated differences for prevenar-specific IgG and IgM levels

	IGG			IGM		
	ED	CI95	P value	ED	CI95	P value
0-4 weeks (A vs P)	19187.8	14098.7 -24276.9	<.0001	13128.5	5356.9 -20900.1	0.0020
4-28 weeks (AP vs PP)	16656.9	11626.7 -21687.1	<.0001	5480.2	1143.1– 9817.3	0.0158
4-28 weeks (AA vs PP)	17155.5	12449.3 -21861.7	<.0001	9272.0	4568.5 -13975.5	0.0005
28-68 weeks (AAA vs PPP)	22733.6	17512.7 -27954.5	<.0001	8189.1	1407.0 -14971.1	0.0209
28-68 weeks (AAP vs PPP)	18439.7	12869.1 -24010.2	<.0001	10320.1	-23.1 -20663.3	0.0505
28-68 weeks (APA vs PPP)	25805.3	19200.6 -32409.9	<.0001	987.0	-6717.0– 8691.0	0.7903
28-68 weeks (APP vs PPP)	17116.7	11720.9 -22512.5	<.0001	2233.9	-4392.3– 8860.1	0.4862

ED=estimated difference, A=active, P=placebo

Figure 1 Study flowchart.

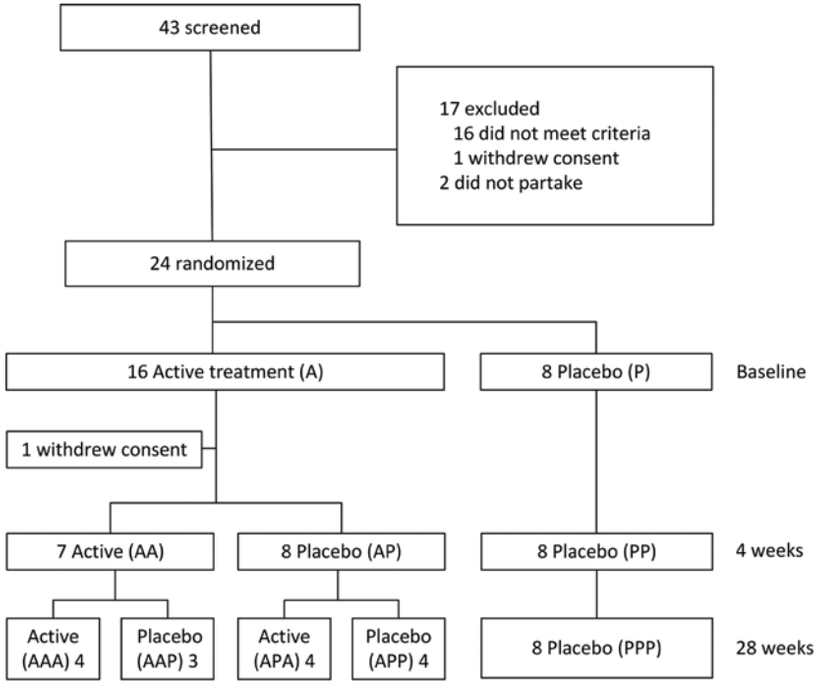


Figure 2 Anti-prevenar (quantified by antibodies against pneumococcal wall saccharide) (A), anti-phosphorylcholine (B) and anti-oxLDL responses (C) Mean + SD. N=4 per group, n=3 for AAP group and n=8 for PPP group. RLU/100ms: relative light units/100ms 'A': active treatment, 'P': placebo treatment. Dotted lines indicate vaccination times (baseline, 4 weeks, 28 weeks). Statistical analysis using a mixed model analysis of variance with fixed factors: treatment group and time, and the interaction of treatment group and subject as random factor.

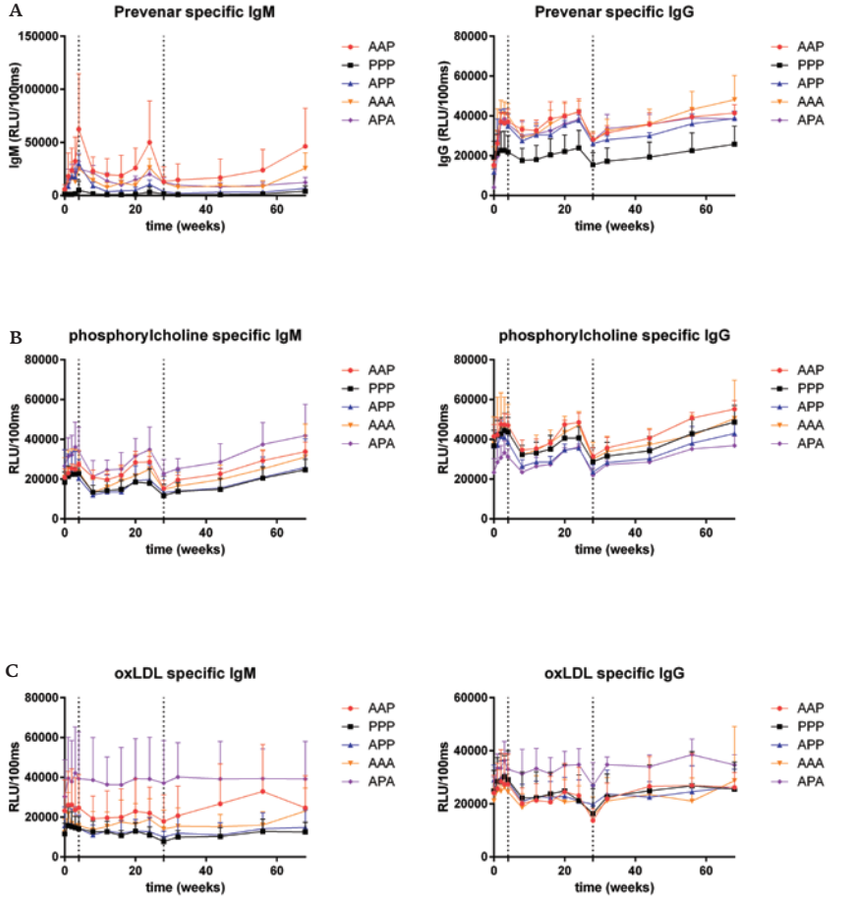
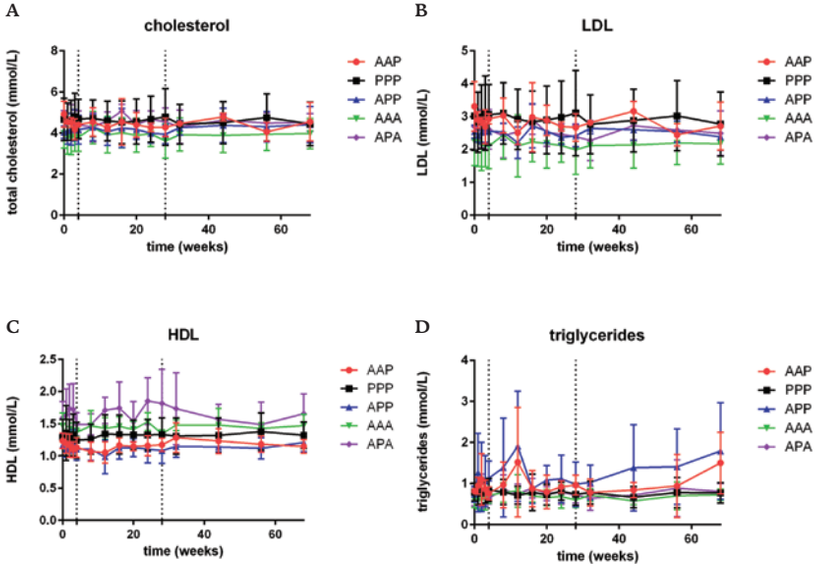


Figure 3 Cholesterol, LDL, HDL and triglycerides plasma levels (mean + SD). N=4 per group, n=3 for AAP group and n=8 for PPP group. 'A': active treatment, 'P': placebo treatment. Dotted lines indicate vaccinations (baseline, 4 weeks, 28 weeks). Statistical analysis using a mixed model analysis of variance with fixed factors: treatment group and time, and the interaction of treatment group and subject as random factor.



All mentioned supplementary figures and tables in this chapter can be found on the corresponding website by scanning this QR code.

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CHAPTER 7

Immunosuppression by hydroxychloroquine: mechanistic proof in *in vitro* experiments but limited systemic activity in a randomized placebo

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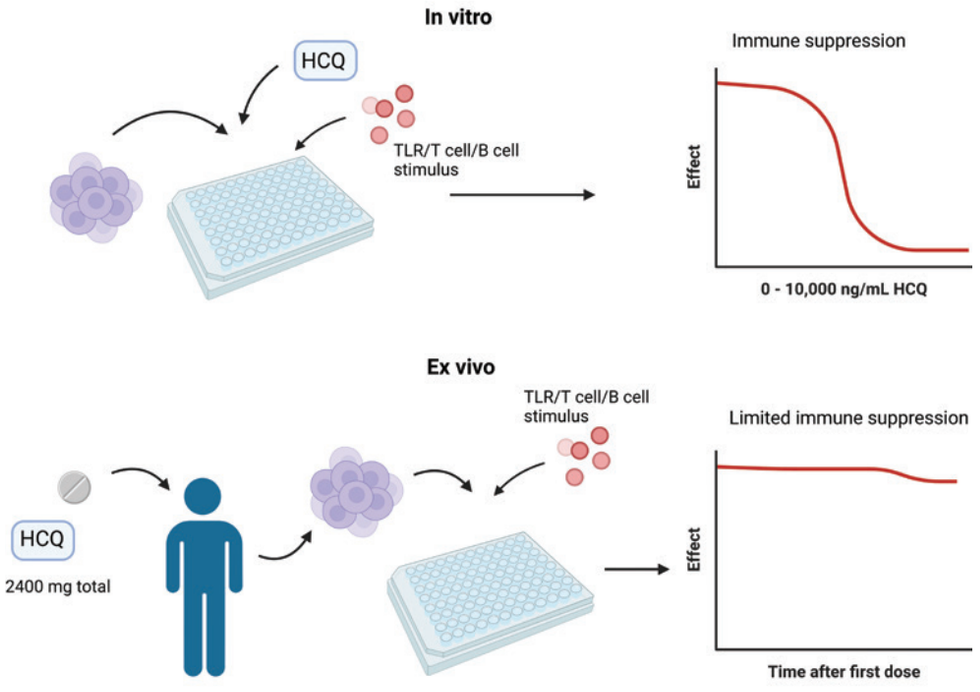
Graphical abstract



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Abstract

Based on its wide range of immunosuppressive properties, hydroxychloroquine (HCQ) is used for the treatment of several autoimmune diseases. Limited literature is available on the relationship between HCQ concentration and its immunosuppressive effect. To gain insight in this relationship we performed *in vitro* experiments in human PBMCs and explored the effect of HCQ on T and B cell proliferation and Toll like receptor (TLR)3/TLR7/TLR9/RIG-I-induced cytokine production. In a placebo-controlled clinical study these same endpoints were evaluated in healthy volunteers that were treated with a cumulative dose of 2400 mg HCQ over 5 days. *In vitro*, HCQ inhibited TLR responses with IC50s >100 ng/mL and reaching 100% inhibition. In the clinical study, maximal HCQ plasma concentrations ranged from 75 to 200 ng/mL. No *ex vivo* HCQ effects were found on RIG-I-mediated cytokine release, but there was significant suppression of TLR7 responses and mild suppression of TLR3 and TLR9 responses. Moreover, HCQ treatment did not affect B cell and T cell proliferation. These investigations show that HCQ has clear immunosuppressive effects on human PBMCs, but the effective concentrations exceed the circulating HCQ concentrations under conventional clinical use. Of note, based on HCQ's physico-chemical properties, tissue drug concentrations may be higher, potentially resulting in significant local immunosuppression.

This trial is registered in the International Clinical Trials Registry Platform (ICTRP) under study number NL8726

Introduction

Hydroxychloroquine (HCQ) is a broad immunosuppressive drug, initially developed as an anti-malarial drug. However, due to its anti-inflammatory properties, HCQ is now widely used in the treatment of autoimmune diseases such as rheumatoid arthritis (RA),[1] systemic lupus erythematosus (SLE)[2] and Sjögren's syndrome.[3] The use of HCQ in other diseases has been under investigation, a pilot trial investigating the use of HCQ in patients after myocardial infarction showed a decrease in plasma IL-6 levels compared to placebo, and a larger trial studying the effect on recurrent cardiovascular events is currently ongoing.[4] Furthermore, HCQ was under investigation for use in moderate to severe COVID-19 patients during the COVID-19 pandemic.[5]

The exact mechanisms behind HCQs immunosuppressive functions remain unclear. HCQ accumulates in the lysosomes and inhibits lysosomal function by autophagosome fusion with lysosomes,[6] thereby inhibiting antigen presentation.[7,8] In addition, HCQ inhibits pro-inflammatory cytokine production by myeloid cells, possibly via the inhibition of endosomal Toll-like receptor (TLR) signalling.[9] It has been shown that HCQ treatment is associated with decreased interferon (IFN)α serum levels in SLE patients.[10] Furthermore, several studies investigating the effect of HCQ on peripheral blood mononuclear cells (PBMCs) or cell lines show that HCQ treatment reduces phorbol 12-myristate 13-acetate (PMA) and ionomycin or lipopolysaccharide induced cytokine production.[11-13]

Besides effects on the innate immune system, HCQ affects the adaptive immune response as well. It has been shown that HCQ inhibits differentiation of class-switched memory B cells into plasmablasts and thereby decreases IgG production in response to TLR9 stimulation or inoculation with inactivated virus.[14,15] HCQ inhibits T cell activation as well, via the inhibition of T cell receptor induced calcium mobilization and dysregulation of mitochondrial superoxide production.[16-18]

However, the concentrations used in such *in vitro* experiments studying the immunomodulatory effects of HCQ largely exceeded obtainable clinical concentrations in patients. A study in cutaneous lupus erythematosus patients receiving HCQ in clinical doses showed that higher HCQ blood levels corresponded with lower *ex vivo* IFNα responses after TLR9 stimulation, but not after TLR7/8 stimulation.[13] Moreover, influenza antibody titers after vaccination in Sjögren's syndrome patients receiving HCQ were lower compared to HCQ naïve patients.[15] Unfortunately, little additional literature is available on the *in vivo* immunomodulatory effects of HCQ and comparing it to *in vitro* experiments.

We aimed to assess and quantify the immunomodulatory effects of HCQ on primary human immune cells, both *in vitro* and *ex vivo* in a randomized clinical trial. We assessed the effect of HCQ on cytokine production after endosomal TLR stimulation in isolated PBMCs and on T and B cell proliferation (*in vitro* as well as *ex vivo*). In the clinical trial, healthy subjects were dosed with HCQ in the standard dosing regimen for moderate-to-severe COVID-19 that was advised in the Netherlands when the study was conceived. In the study design, we accounted for a potential age effect on the study outcomes, since general immunocompetence and drug metabolism has been reported to be age-dependent.[19,20] Here we present the outcomes of the *in vitro* experiment and the randomized clinical trial.

Methods

IN VITRO EXPERIMENTS

Blood was collected by venipuncture using Sodium Heparin vacutainer tubes or Cell Preparation Tubes (CPT, Becton Dickinson, Franklin Lakes, NJ, USA) from healthy volunteers after written informed consent, in accordance with Good Clinical Practice guidelines and the Declaration of Helsinki. Blood was used for the evaluation of the *in vitro* immunomodulatory activity of hydroxychloroquine (10 - 10,000 ng/mL, Sigma-Aldrich, Deisenhofen, Germany). All experiments were started within one hour after blood withdrawal, and incubations were performed in duplicate. Hydroxychloroquine and stimulant were added simultaneously. Per experiment, blood of 6 donors was used.

CLINICAL STUDY

We conducted a single-blind, randomized, placebo-controlled multiple dose study in forty healthy male volunteers, comprising twenty young (18-30 years) and twenty elderly (65-75 years) subjects. The study was conducted at the Centre for Human Drug Research in Leiden, The Netherlands,

between June and September 2020, during the COVID-19 pandemic. All subjects in the clinical trial gave written informed consent according to Declaration of Helsinki recommendations, prior to any study-related activity. The study was approved by the Independent Ethics Committee of the Foundation 'Evaluation of Ethics in Biomedical Research' (Stichting Beoordeling Ethiek Biomedisch Onderzoek, Assen, The Netherlands) and registered in the Toetsingonline Registry (study number NL73816.056.20), and in the International Clinical Trials Registry Platform (NL8726).

VOLUNTEER SELECTION

To avoid sex-related inter-individual variability in immune responses, only male subjects were included.[21] Subjects were included if they were overtly healthy. The health status of subjects was assessed by medical screening, including medical history, physical examination, vital signs measurements, 12-lead electrocardiography (ECG), urine analysis, drug screen and safety chemistry, coagulation, and hematology blood sampling. BMI of study participants had to be between 18 and 32 kg/m^2. Subjects with a known hypersensitivity reaction to chloroquine, HCQ or other 4-aminoquinolines, abnormalities in the resting ECG (including QTcF-interval >450ms), evidence of any active or chronic disease or condition (including long QT syndrome, retinal disease, G6PD deficiency, autoimmune diseases, diabetes mellitus type I or II, psychiatric disorders) or a positive SARS-COV-2 PCR test were excluded from study participation. Use of concomitant medication was not permitted during the study, and 14 days (or 5 half-lives) prior to the study drug administration, except for paracetamol.

STUDY DESIGN

Subjects were randomized to receive either hydroxychloroquine sulphate (plaquenil®) or placebo tablets, in a 1:1 ratio. Tablets were dispensed by the pharmacy, according to a randomization list generated by a study-independent statistician. Plaquenil® and placebo tablets were packaged in the same way but the tablets were not indistinguishable, study drug administration was therefore performed by dedicated unblinded personnel not involved in any other study tasks. Subjects received HCQ or placebo by a loading dose of 400 mg twice daily (t=0h and t=12h) followed by a 400 mg once daily dose regimen (t=24h, t=48h, t=72h, and t=96h), giving a cumulative dose of 2400 mg. This reflected the standard dosing regimen for moderate-to-severe COVID-19 patients in the Netherlands when the study was conceived (total dose between 2000 and 3800 mg).

PHARMACOKINETIC EVALUATION

For pharmacokinetic (PK) assessments, blood was collected in 3 mL Vacutainer® K_2EDTA tubes (Becton Dickinson) on study day 0 (baseline and 3 hours post-dosing), and day 1, 4 and 9 (3 hours post-dosing). Hydroxychloroquine plasma concentrations were measured by Ardena Bioanalytical Laboratory (Assen, the Netherlands) using a validated LC-MS/MS method. The lower limit of quantification (LLOQ) of the analysis was 5 ng/mL.

WHOLE BLOOD STIMULATION

Whole blood was stimulated with 10 µg/mL phytohemagglutinin (PHA, Sigma-Aldrich) for 6 hours and 24 hours. After 6 hours, activation markers on T-cells were measured using CD69-APC (clone: REA824), CD71-FITC (clone: REA902), CD154-VIOBLUE (REA238) and CD25-PE (clone: 3G10), CD3-VioGreen (REA613), CD4-APC-VIO770 (REA623) and CD8-PE-VIO770 (REA734) antibodies and propidium iodide as viability dye (all Miltenyi Biotec, Bergisch-Gladbach, Germany) using a MACSQuant 16 analyzer (Miltenyi Biotec). After 24 hours, culture supernatants were collected for cytokine analysis.

PBMC ISOLATION AND TLR STIMULATION

PBMCs were isolated from CPT after centrifugation at 1800 x g for 30 minutes, and washed 2x using phosphate buffered saline (PBS, pH 7.2, Gibco, Thermo Fisher, Waltham, MA, USA). PBMCs were stimulated with endosomal TLR ligands PolyI:C (TLR3, 50 µg/mL), imiquimod (TLR7, 1 µg/mL), CpG class A (TLR9, oligodeoxynucleotides [ODN] 2.5 µM) and POLYI:C/lyovec (RIG-I, 1 µg/mL; all Invivogen, Toulouse, France). Supernatants were collected after 24 hours for cytokine quantification.

PROLIFERATION ASSAY

PBMCs were stained with 2.5µM cell trace violet (CTV, Thermo Fisher) according to user's manual. T cells were stimulated with 5 µg/mL phytohemagglutinin (PHA), and B cells with a monoclonal CD40 antibody (5 µg/mL; clone: G28.5, BioXCell) and CpG class B (2.5 µM; ODN Invivogen). After 5 days

of stimulation PBMCs were stained using, CD4-PE (clone: OKT4), CD8-APC (clone: HIT8A), CD19-PE (clone: HIB19, all Biolegend, San Diego, CA, USA) and fixable viability dye eFluor780 (Thermo Fisher) and proliferation was quantified by flow cytometry, using the MACSQuant 16 analyzer.

FLOW CYTOMETRY

Circulating leukocyte subsets were analyzed using flow cytometry. Red blood cell lysis was performed on sodium heparinized blood using RBC lysis buffer (Thermo Fisher Scientific). After washing with PBS (PH 7.2), leukocytes were incubated with fluorochrome-labeled antibodies for 30 minutes on ice. After a final washing step, leukocytes were measured on a MACSQuant 16 analyzer (Miltenyi Biotec). See supplemental table I for a full list of antibodies used.

CYTOKINE MEASUREMENTS

IFNγand IL-2 were quantified using the Vplex-2 kit (Meso Scale Discovery). IFNα and IL-6 were quantified using the pan-specific IFNα ELISAPRO HRP kit and the IL-6 ELISAPRO HRP kit (both Mabtech, Nacka Strand, Sweden).

STATISTICAL ANALYSIS

In vitro data are reported as mean ± standard deviation (SD). The IC50 was calculated using a inhibitory sigmoid Emax function where applicable. Analyses were performed using Graphpad Prism version 6.05 (Graphpad, San Diego, CA, USA).

Repeatedly measured pharmacodynamic data were evaluated with a mixed model analysis of variance with fixed factors treatment, age group, time, treatment by time, age group by time, treatment by age group and treatment by age group by time and a random factor subject and the average pre-value as covariate. If needed, variables were log transformed before analysis. Contrasts between the placebo and HCQ treatment groups were calculated per endpoint. In addition, a potential age-specific HCQ effect was evaluated by comparing the 18-30 years with the 65-75 years age group. For the contrasts, an estimate of the difference (back-transformed in percentage for log transformed parameters), a 95% confidence interval (in percentage for log-transformed parameters), Least Square Means (geometric means for log transformed parameters), and the p-value were calculated. A P-value ≤ 0.05 was considered to be statistically significant.

All calculations were performed using SAS for windows V9.4 (SAS Institute, Inc., Cary, NC, USA).

Results

HYDROXYCHLOROQUINE SUPPRESSED ENDOSOMAL TLR-INDUCED IFNα AND IL-6 RELEASE IN VITRO

PBMCs were stimulated with endosomal TLR ligands in the presence of a dose range of HCQ for 24 hours, and supernatants were analyzed for IRF-mediated IFNα and for NFκB-mediated IL-6 secretion. PBMCs were stimulated with different endosomal TLR ligands: POLY I:C (TLR3), imiquimod (TLR7), CpG class A (TLR9) and POLY I:C lyovec (RIG-I). HCQ dose-dependently inhibited endosomal TLR-induced IFNα and IL-6 secretion (Figure 1). POLY I:C-induced IFNα and IL-6 release was strongly suppressed at 10.000 ng/mL (IFNα: -83.9%, IL-6: -96.6%, IC50 IL-6=637.2 ng/mL). Imiquimod (IMQ)-induced cytokine release was completely suppressed at the highest concentration (IFNα: -96.3%, IL-6: -96.3%, IC50 IFNα: 695.8 ng/mL, IL-6: 237.9 ng/mL). The same was observed for stimulation with CpG class A, IFNα was suppressed by 99.6% with an IC50 of 145.3 ng/mL, and IL-6 was suppressed by 96.4%, with an IC50 of 86.9 ng/mL. The RIG-I response to POLY I:C/lyovec was less affected by HCQ, while IFNα release was suppressed by 66.1% at 10,000 ng/mL HCQ, IL-6 release was not significantly altered.

HCQ INHIBITED B CELL PROLIFERATION BUT NOT T CELL PROLIFERATION IN VITRO

PBMCs were stimulated with phytohaemagglutinin (PHA) or monoclonal anti-CD40 with CpG-B to induce T cell and B cell proliferation respectively, in the presence of a dose range of HCQ. No effect of HCQ was seen on T cell proliferation (Figure 2A). Also, no effects were observed on T cell activation markers after PHA stimulation for 6 hours (Figure S1). At HCQ concentrations >100 ng/mL, a decrease in B cell proliferation was observed, with an IC50 of 1138 ng/mL (Figure 2B).

CLINICAL STUDY

Demographics and safety

Of the 40 enrolled and randomized healthy subjects, 20 received a cumulative dose of 2400 mg HCQ in 5 days and 20 received placebo (Figure 3). The

different age groups (18–30 and 65–75 years) were of equal size. Baseline characteristics are described in Table 1. All subjects completed their study treatment. One subject in the 65–75 years group erroneously took an additional 400 mg dose of HCQ on study day 2, after which the subject received 400 mg doses (once daily) for two consecutive days to not exceed the cumulative dose of 2400 mg.

Treatment-emergent adverse events were transient, of mild severity and did not lead to study discontinuation. Adverse events were reported more often by subjects in the active treatment arm (50%) compared to placebo (35%). Gastrointestinal complaints (20%) and dizziness (15%) were the most frequently reported adverse events in the active group. There were no findings of clinical concern following assessments of urinalysis, hematology and chemistry laboratory tests, vital signs, physical examination and ECGs.[22]

Pharmacokinetics

Mean HCQ concentration time profiles in plasma are depicted in Figure 4A. Individual concentration profiles have been published previously.[22] There were no significant differences in HCQ exposures between age groups (Figure 4B). Mean concentrations measured 27 hours after starting the treatment course (day 1, 121.0 ± 40.54 ng/mL) were in a similar range to those measured on the last day of the treatment course (day 4, 109.2 ± 35.59 ng/mL).

PHARMACODYNAMICS

Hydroxychloroquine did not affect circulating immune cells

The effects of HCQ on different circulating cell populations, both absolute as relative, were evaluated using flow cytometry. No apparent effects were seen on absolute values of total leukocytes, lymphocytes, monocytes or neutrophils (Table S2), as well as $CD14^+$ monocytes, $CD19^+$ B cells, $CD3^+$ T cells, $CD4^+$ T cells and $CD8^+$ T cells (Table S3). Furthermore, no effects were seen on relative T cell populations ($CD3^+$) in general, nor on subpopulations of T helper cells ($CD4^+$), cytotoxic T cells ($CD8^+$), and regulatory T cells ($CD4^+CD25^+CD127^-$). Similarly, no apparent treatment effects were observed in natural killer cells ($CD56^+$), B cells ($CD19^+$) and subpopulations of regulatory ($CD5^+CD1d^{hi}$), transitional ($CD24^{hi}CD38^{hi}$) and antibody secreting B cells ($CD27^+CD38^+$). Moreover, also in classical ($CD14^+$), non-classical ($CD16^+$) and intermediate ($CD14^+CD16^+$) monocytes and plasmacytoid dendritic cells (pDCs, $HLA\text{-}DR^+CD14^-CD16^-CD123^+$) no differences were found between treatment groups. Also, between both age groups, no evident HCQ effects were observed (Table S3).

In vivo hydroxychloroquine suppressed IFNα secretion following TLR7 stimulation, but not after TLR3, TLR9 or RIG-I-like receptor stimulation

To study the effects of HCQ on TLR/RIG-I-mediated IRF activation, PBMCs were stimulated with different endosomal TLR ligands: POLY I:C (TLR3), imiquimod (TLR7), CpG class A (TLR9) and POLY I:C lyovec (RIG-I). Overall, no HCQ effect was observed on IFNα responses (Figure 5), except for a significant suppression of IMQ-driven IFNα production (inhibition of -48.2%, CI95 -72.1%– -4.0%, p=0.038). POLY I:C-driven IFNα release also appeared to be suppressed by HCQ, but not significantly (inhibition -34.2%, CI95 -57.7% - 7.5%, p=0.091). No differences in HCQ effect on IFNα responses were observed between the young and elderly population (Figure S3).

In vivo hydroxychloroquine significantly suppressed IL-6 secretion after TLR7 stimulation, but not following TLR3, TLR9 or RIG-I-like receptor stimulation

Activation of NFκB signaling via endosomal TLR and RIG-I-like ligands was assessed by measuring downstream IL-6 production (Figure 6). HCQ significantly suppressed IMQ-driven IL-6 production (inhibition of -71.3%, CI95 -84.7%– -46.1%, p=0.0005). No significant HCQ effects were observed on IL-6 production driven by CpG A (TLR9) and POLY I:C (TLR3) stimulations (inhibition of -35.9%, CI95 -60. 3%– 3.6%, P=0.068 and -37.7%, CI95 -62.6% - 3.7%, P=0.067, respectively). No differences in HCQ effect on IL-6 responses were observed between the young and elderly population (Figure S3).

In vivo hydroxychloroquine did not alter T cell activation

To further investigate the potential immunomodulatory effect of HCQ on T cell activation, whole blood samples were incubated with PHA, which is known to induce a general T cell response.[23] HCQ treatment did not modulate expression of T cell activation markers (CD25, CD69, CD71, CD154) following PHA-stimulation (Figure S3). In addition, PHA-induced secretion of IL-2 and IFNγwas assessed, no apparent differences were observed between HCQ and placebo (Figure S4).

Hydroxychloroquine did not alter ex vivo B and T cell proliferation after in vivo administration

Proliferative capability of B cells was assessed by stimulating PBMCs *ex vivo* with anti-CD40 mAb + CpG B ODN, a known stimulus for human B cell activation.[24] Following stimulation of PBMCs, the percentage of proliferative B cells in the HCQ-treated group was similar to that of the placebo group (70.47% at day 4 for placebo, 70.03% for HCQ) (Figure 7). In addition, PBMCs were stimulated with PHA to induce T helper cells ($CD4^+$) and cytotoxic T cells ($CD8^+$) proliferation. Proliferation of both $CD4^+$ and $CD8^+$ cells was comparable between the HCQ- and placebo-treated group (>95% for both groups for all time points for CD4, >92% for both groups for all time points for CD8). No differences were observed for B and T cell proliferation in the separate age groups (Figure S5).

Discussion

Although HCQ is widely used for the treatment of autoimmune diseases, the exact mechanism behind its immunomodulatory properties remains unclear. In this study we therefore aimed to quantify the immunosuppressive effect of HCQ by studying the endosomal TLR response and lymphocyte proliferation and activation both in *in vitro* experiments and *in vivo* in a randomized placebo-controlled trial in healthy volunteers.

In our *in vitro* experiments, HCQ dose-dependently inhibited TLR3-, 7- and 9-driven IL-6 and IFNα production, with profound effects at concentrations >100 ng/mL. These findings are in line with literature on TLR signaling modulation by chloroquine. [9,25] Limited data are available on the immunomodulatory effect of HCQ/chloroquine on RIG-I signaling.[26] RIG-I functions as a cytosolic sensor of nucleic acids, inducing a type I IFN response after activation. HCQ inhibited the IFN responses in THP-1 cells transfected with RIG-I ligands,[27] but this effect was not confirmed in cultures of human bronchial smooth muscle and epithelial cells.[28,29] This is in line with the observations in the current study, which shows that HCQ only mildly modulated RIG-I-mediated IFNα production in PBMCs, without affecting IL-6 release. Our results suggest that HCQ has a profound effect on endo-lysosomal TLR functioning *in vitro* but affects the cytosolic RIG-I-mediated pathway to a lesser degree. This could be explained by HCQ's excessive affinity to the lysosomal intracellular compartment (expected to be 56,000-fold higher than cytosol).[30]

HCQ did not affect T cell activation *in vitro*. Although a dose-dependent inhibition of T cell proliferation by chloroquine following stimulation with anti-CD3/CD28 has been described,[31-33] we did not see any inhibitory effect of HCQ on T cell proliferation or expression of activation markers in our *in vitro* experiments. This may be explained by the fact that a different and more potent stimulus was used in this study (PHA), which might be more difficult to suppress. For B cell proliferation, on the other hand, a dose-dependent HCQ-mediated inhibition was observed *in vitro*, confirming previous research.[34] Although the HCQ-mediated inhibition was not as strong as the inhibition of cytokine production (IC50 of 1138 ng/mL for B cell proliferation vs 145-696 ng/mL for cytokine production), at concentrations > 100 ng/mL a clear HCQ-mediated decrease in B cell proliferation was found.

While HCQ had strong immunosuppressive effects *in vitro*, especially at high concentrations, less pronounced *ex vivo* effects of the compound were observed in our clinical study. Compared to placebo, 5-day HCQ treatment did not significantly suppress B cell proliferation or *ex vivo* TLR-driven IFNα and IL-6 secretion in PBMC cultures, except for a suppressive effect on TLR7-driven responses.The most likely explanation for this discrepancy between *in vitro* and *ex vivo* is that there was insufficient drug exposure at the evaluated HCQ dose and regimen in the clinical study. By using a 5-day dose regimen of HCQ (the recommended off-label dose for COVID-19 at the time of study conduct), an average maximum plasma concentration of 121 ng/mL was reached. This concentration is considerably lower than plasma levels found in RA patients receiving HCQ treatment of 200 mg daily for a longer time period, which ranges from 200–500 ng/mL.[35–37] Peak exposures of 100-150 ng/mL from the clinical study translate into a maximal inhibitory effect of 20 to 50% in most cellular assays. In combination with the observed variability of the endpoints, such effects remain easily undetected. However, whole blood concentrations are expected to be approximately 2-to-7-fold higher than plasma concentrations due to intracellular uptake in blood components,[38-40] which would make the concentrations more in range with the *in vitro* experiments. Also, due to the large volume of distribution,[39] and the high HCQ tissue concentrations as compared to plasma,[41,42] immunosuppressive effects in specific tissues may be significant. Moreover, HCQ has a gradual onset of action for HCQ, and is biologically active even after drug discontinuation.[8] This would mean that the five-day treatment that was used in the current study is insufficient to detect *ex vivo* drug effects. Other studies, for

example investigating HCQ effect in HIV patients,[43] showed a discrepancy between plasma levels and drug efficacy.

The widespread use of hydroxychloroquine following the onset of the COVID-19 pandemic was the reason to initiate our experiments. The initial off-label use of HCQ was primarily based on studies that assessed *in vitro* antiviral activity against SARS-COV-2.[44] However, there is also a long-standing hypothesis that the immunomodulatory properties of chloroquine and HCQ could dampen immunopathology caused by viral infections such as influenza, Severe Acute Respiratory Syndrome (SARS), Middle East Respiratory Syndrome (MERS) and COVID-19 by suppressing the host immune response.[45-47] Use of HCQ in COVID-19 patients did not show evident favorable effects for clinical endpoints such as mortality and mechanical ventilation for both prophylaxis and treatment.[48] Our study provides mechanistic insight in the immuno-modulatory effects of a HCQ dosing regimen that was used to treat COVID-19. We found that a 5-day treatment course of HCQ did not have extensive immuno-modulatory effect in healthy individuals. HCQ treatment only significantly inhibited TLR7 responses. In theory, inhibition of the TLR7-mediated innate response to viral agents may be disadvantageous during the initial stages of viral infection.[49,50] However, recent COVID-19 trials did not show an effect of HCQ treatment on disease incidence, and long-term HCQ use in rheumatoid arthritis is not associated with higher incidence of upper respiratory tract infections.[51,52]

In conclusion, we showed extensive and profound immunomodulation by HCQ *in vitro*, however in a clinical study in healthy volunteers, the overall immunomodulatory effects of a 5-day HCQ treatment regimen of 2400 mg were limited. The pharmacological activity of HCQ in autoimmunity remains to be studied in greater detail, based on the assays as presented in our studies and at a therapeutic dose and regimen relevant for the condition of interest.

Table 1 Baseline characteristics.

	Hydroxychloroquine		Placebo	
	Age group 18–30 yrs (n=10)	Age group 65–75 yrs (n=10)	Age group 18–30 yrs (n=10)	Age group 65– 75 yrs (n=10)
Age, median (range)	23 (20–26)	68 (65–70)	23 (18–25)	68 (65–71)
BMI, mean (SD)	21.8 (1.5)	25.8 (2.0)	24.4 (1.9)	24.2 (3.0)
Race or ethnicity,*n (%)				
White	10 (100)	10 (100)	10 (100)	10 (100)
Other	0 (0)	0 (0)	0 (0)	0 (0)

**Self-reported race or ethnicity of subjects. BMI=body mass index; SD=standard deviation.*

Figure 1 HCQ dose-dependently inhibited endosomal TLR induced IFNα and IL-6 release in vitro. PBMCs were stimulated with 50 µg/mL POLYI:C (TLR3), 1 µg/mL IMQ (TLR7), 2.5 µM CpG-A (TLR9) or 1 µg/mL POLY I:C/lyovec (RIG-I) for 24 hours in the presence of a dose range of HCQ. IFNα and IL-6 release were measured by ELISA. The mean ± SD of the change from baseline of 6 subjects is shown. The IC50 was calculated using a four-parameter non-linear regression fit where applicable.

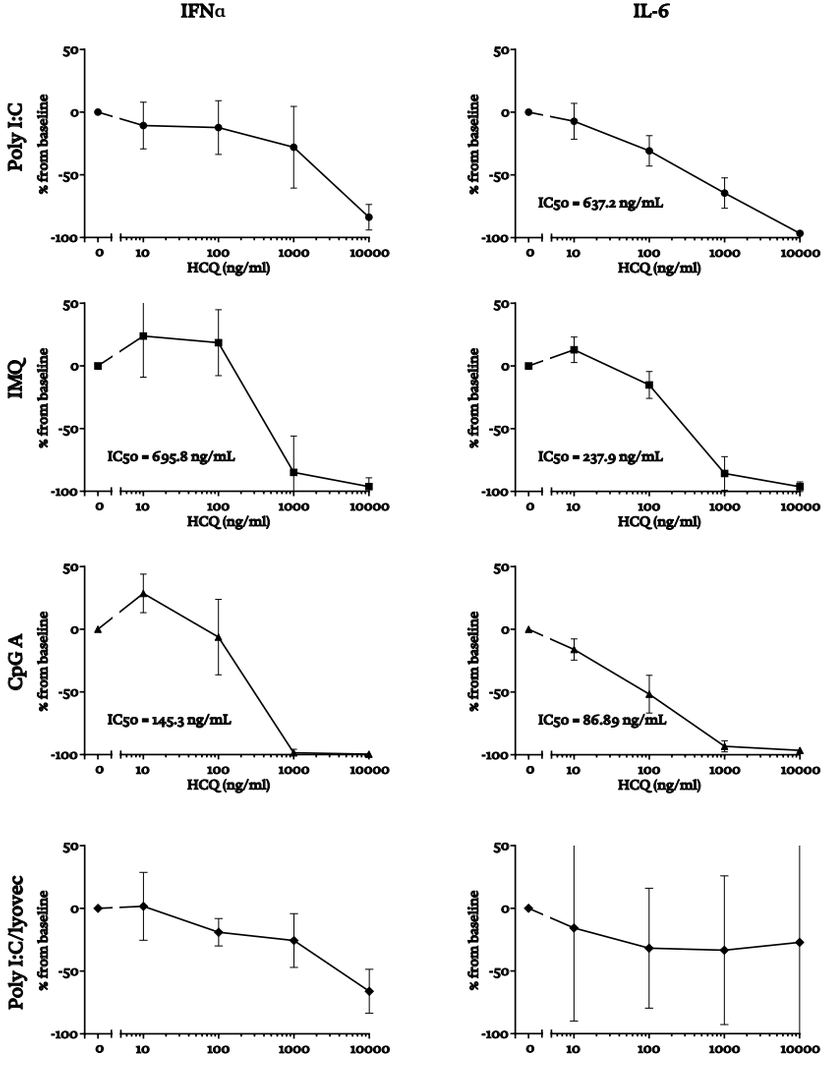


Figure 2 HCQ dose-dependently inhibited B cell, but not T cell proliferation in vitro. PBMCs from 6 healthy donors were stained with CTV and stimulated for 5 days with 5µg/ml PHA for T cell proliferation (A), or 5 µg/mL anti-CD40 MAB + 2.5 µM CpG B for B cell proliferation (B). Proliferation was measured by flow cytometry. The mean ± SD of the change from baseline are shown. The IC50 was calculated using a four-parameter non-linear regression fit where applicable.

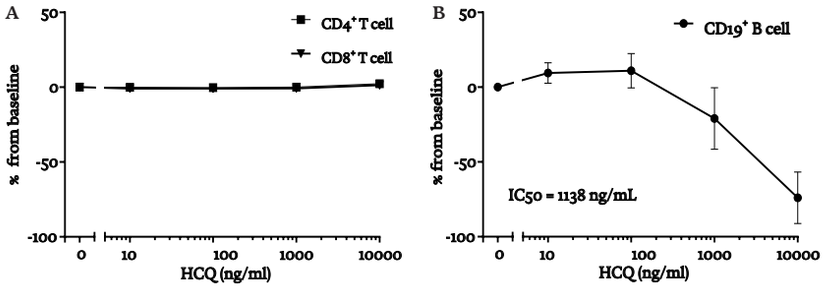
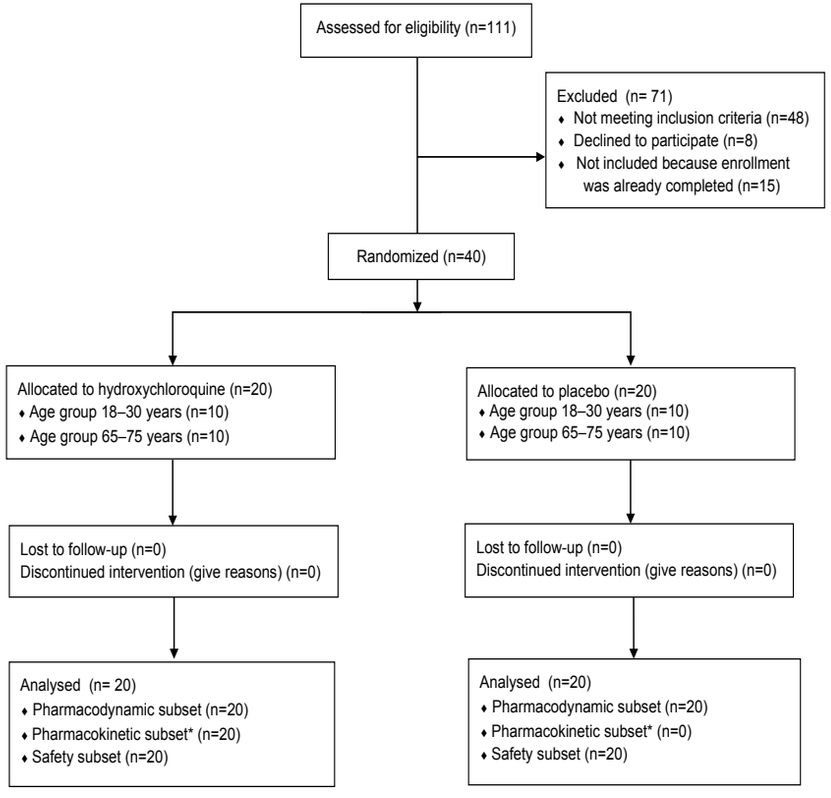


Figure 3 Trial flow chart (CONSORT diagram).



** Drug concentrations were only analyzed in the active treatment group.*

Figure 4 Pharmacokinetic profile of HCQ. Mean and standard deviation of hydroxychloroquine plasma concentrations for HCQ treatment group (A), and split for young and elderly volunteers (B). Dotted vertical lines indicate timing of HCQ dosing (0, 12, 24, 48, 72, 96 hrs).

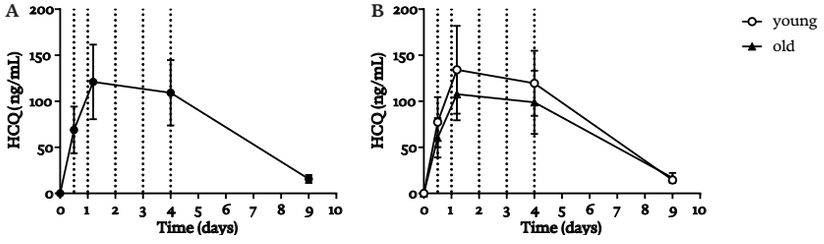


Figure 5 In vivo HCQ inhibited IMQ-induced IFNα release, but not TLR3, TLR9 and RIG-I. PBMCs were stimulated with 50 µg/mL poly I:C (TLR3), 1 µg/mL IMQ (TLR7), 2.5µM CpG A (TLR9) or 1 µg/mL poly I:C/lyovec (RIG-I) at 0, 12, 24, 48, 72 and 92 hours after primary HCQ dosing. IFNα release was measured by ELISA. Data is shown as mean + SD as one-sided error bars. Dotted vertical lines indicate HCQ dosing times.

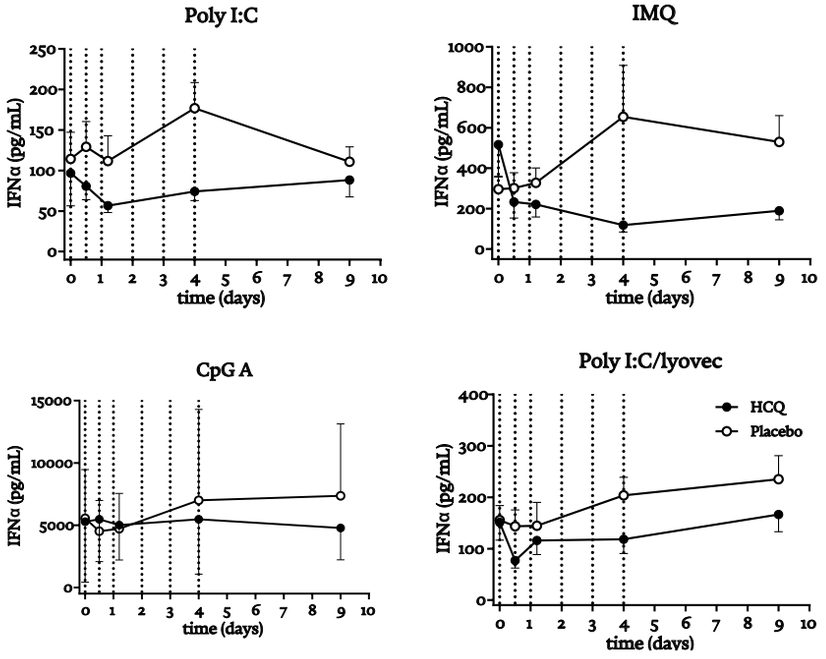


Figure 6 In vivo HCQ inhibited IMQ-induced IL-6 release, but not TLR3, TLR9 and RIG-I. PBMCs were stimulated with 50 µg/mL poly I:C (TLR3), 1 µg/mL IMQ (TLR7), 2.5µM CpG A (TLR9) or 1 µg/mL poly I:C/lyovec (RIG-I) at 0, 12, 24, 48, 72 and 92 hours after primary HCQ dosing. IFNα release was measured by ELISA. Data is shown as mean + SD as one-sided error bars. Dotted vertical lines indicate HCQ dosing times.

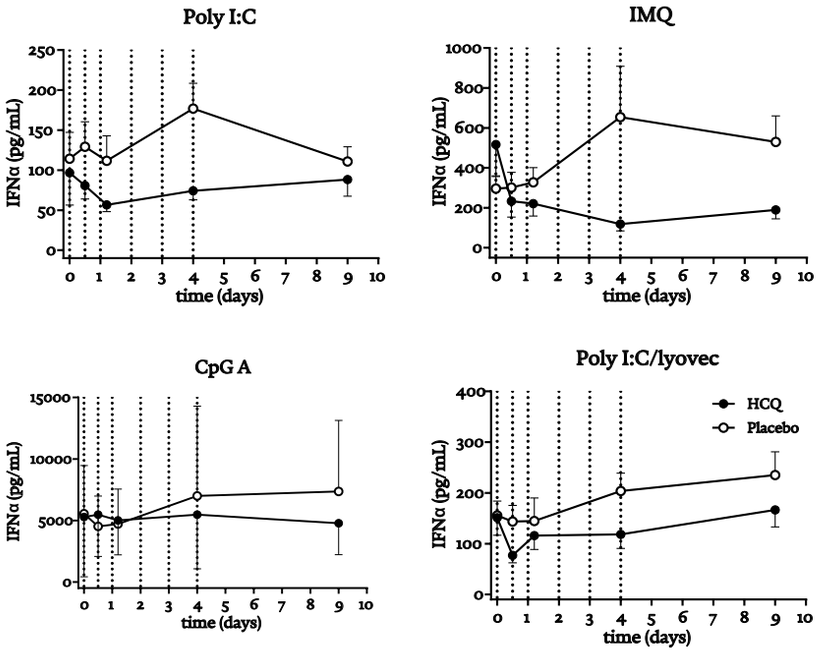
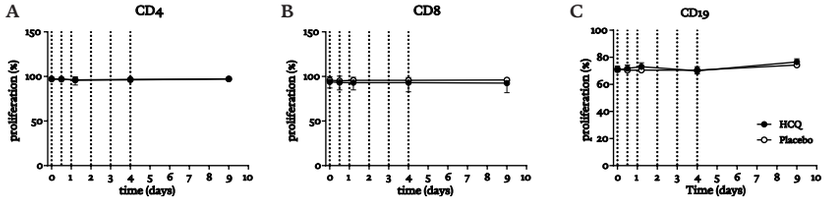


Figure 7 In vivo HCQ did not affect T and B cell proliferation. PBMCs were stained with CTV and stimulated for 5 days with 5µg/ml PHA for T cell proliferation (A), or 5 µg/mL anti-CD40 MAB + 2.5 µM CpG B for B cell proliferation (B). Proliferation was measured by flow cytometry. The mean ± SD are shown. Dotted vertical lines indicate HCQ dosing times.



All mentioned supplementary figures and tables in this chapter can be found on the corresponding website by scanning this QR code.

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CHAPTER 8

OX40L inhibition suppresses KLH-driven immune responses in healthy volunteers: a randomized controlled trial demonstrating proof-of-pharmacology for KY1005

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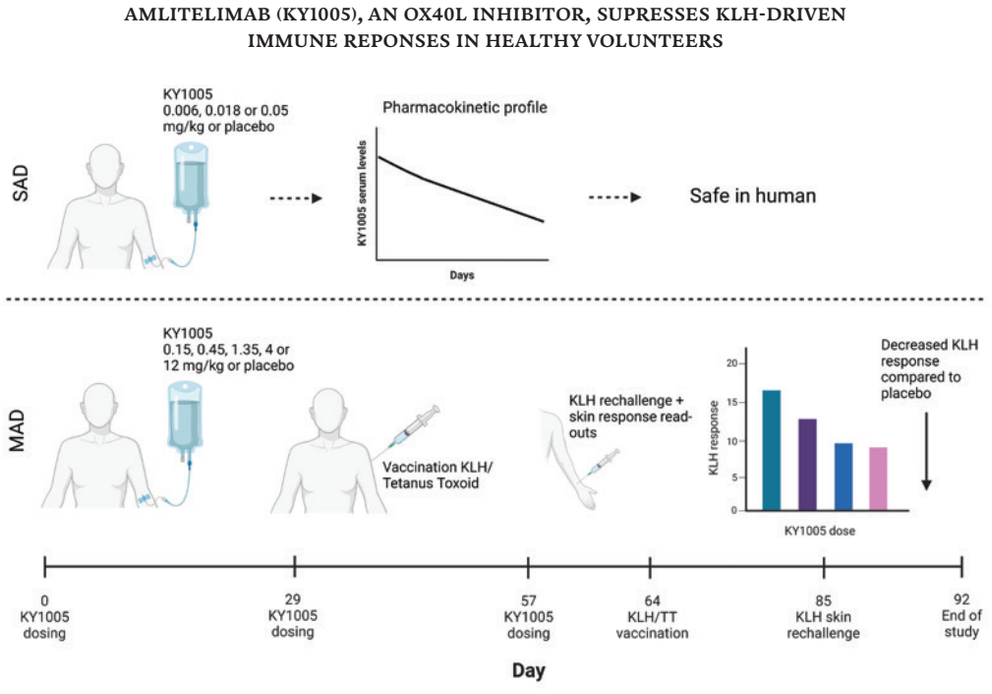
Graphical abstract



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Abstract

The safety, tolerability, immunogenicity and pharmacokinetic (PK) profile of an anti-OX40L monoclonal antibody (KY1005) were evaluated. Pharmacodynamic (PD) effects were explored using keyhole limpet haemocyanin (KLH) and tetanus toxoid (TT) immunizations.

Sixty-four healthy male subjects (26.5±6.0 years) were randomized to single doses of 0.006, 0.018, 0.05mg/kg or multiple doses of 0.15, 0.45, 1.35, 4, 12mg/kg KY1005 or placebo (6:2). Serum KY1005 concentrations were measured. Antibody responses upon KLH and TT immunizations and skin response upon intradermal KLH administration were performed. PD data was analysed using repeated measures ANCOVAs and post-hoc exposure-response modelling.

No serious adverse events occurred and all adverse events were temporary and of mild or moderate severity. A non-linear increase in mean serum KY1005 concentrations was observed (median t_{max} ~4 hours, geometric mean t½ ~24 days). Cutaneous blood perfusion (estimated difference (ED) -13.4 arbitrary unit (AU), 95% CI -23.0 AU– -3.8 AU) and erythema quantified as average redness (ED -0.23 AU, 95% CI -0.35 AU– -0.11 AU) decreased after KY1005 treatment at doses of 0.45mg/kg and above. Exposure-response analysis displayed a statistically significant treatment effect on anti-KLH antibody titres (IgG Emax -0.58 AU, 95% CI -1.10 AU– -0.06 AU) and skin response (erythema Emax -0.20 AU, 95% CI -0.29 AU– -0.11 AU).

Administration of KY1005 demonstrated an acceptable safety and tolerability profile and PK analyses displayed a non-linear profile of KY1005. Despite the observed variability, skin challenge response after KY1005 treatment indicated pharmacological activity of KY1005. KY1005 shows potential as a novel pharmacological treatment in immune mediated disorders.

Introduction

The significance of the T cell co-stimulatory molecule OX40 and its ligand OX40L in immunoregulation is increasing, especially as therapeutic targets. OX40 is predominantly expressed on activated memory and regulatory cluster of differentiation 4 ($CD4^+$) T cells and in lower levels on activated $CD8^+$ T cells,[1] natural killer cells,[2,3] and neutrophils.[4] OX40 agonism has been shown to result in an increase of the antigen-specific T cell pool[5,6] and prolonged activation.[7] Similar to OX40, the expression of OX40L is upregulated after antigen presentation on various antigen-presenting cells such as B cells,[8] dendritic cells,[9] macrophages[10] and specific cell types outside the immune system.[11–14] Activation of this co-stimulatory OX40-OX40L pathway may contribute to resistance of T-lymphocytes to regulatory signals.[15]

OX40-OX40L signalling may be a target for the treatment of auto-immune diseases.[16–21] Several animal models confirmed that OX40L is involved in diabetes,[22] colitis,[23] rheumatoid arthritis,[24] uveitis[25] and multiple sclerosis.[26,27] In human studies, OX40 inhibition using an anti-OX40 monoclonal antibody has been shown to improve the Eczema Area and Severity Index (EASI) score in atopic dermatitis patients with up to 56% reduction from baseline EASI score compared to 38% reduction in placebo treated subjects.[20] Another study showed that although OX40L inhibition with an anti-OX40L monoclonal antibody in patients with mild allergic asthma had no effect on airway hyperresponsiveness or allergen-induced airway responses however total serum immunoglobulin E (IgE) decreased 16.5% from baseline compared to a 14% increase in placebo treated subjects and sputum eosinophils decreased 75% from baseline compared to a 14% decrease in placebo treated subjects.[28] Blockade of the OX40-OX40L pathway seems a scientifically plausible approach to modulate persistent inflammation caused by autoreactive memory T effector cell populations. This blockade may possibly also induce or restore immune tolerance to autoantigens (e.g., in autoimmune disease) or alloantigens (e.g., following transplants).

KY1005 is a novel non-depleting IgG4 human anti-OX40L monoclonal antibody that binds OX40L and thereby prevents persistent inflammation by blocking the interaction with OX40. *In vitro,* KY1005 inhibited interleukin 2 (IL-2), IL-13 and tumour necrosis factor α release in human mixed lymphocyte reaction (MLR) tests (Supplementary Materials and Methods S1). *In vivo* studies in rhesus monkeys with acute Graft versus Host Disease showed prolonged median survival time (MST) >100 days when KY1005 was co-administered with sirolimus compared to KY1005 monotherapy (MST 19.5 days), sirolimus monotherapy (MST 14 days) or no prophylaxis (MST 8 days).[29] The synergistic effect of KY1005/sirolimus was possibly induced by sustained T regulatory cell reconstitution as well as suppression of T effector activity. Based on these experiments, KY1005 could be a treatment modality to inhibit the activation of the immune system as a result of high OX40-OX40L expression and consequently restore the homeostasis between proinflammatory T effector and anti-inflammatory T regulatory cells in immune mediated diseases.

The aim of this first-in-man study was to evaluate the safety and tolerability, immunogenicity and pharmacokinetic (PK) profile of KY1005 in healthy volunteers. Intramuscular immunizations with a neoantigen (keyhole limpet haemocyanin, KLH) and a recall antigen (tetanus toxoid, TT) were used to explore pharmacodynamic (PD) effects of KY1005, including measurements of serum anti-KLH and anti-TT antibody titres and objective quantification of skin challenge response following an intradermal KLH administration.

Materials and methods

This was a phase 1, randomized, placebo-controlled, double-blind, single (SAD) and multiple ascending dose (MAD) study in sixty-four (64) healthy volunteers performed at the Centre for Human Drug Research (CHDR), Leiden, The Netherlands. The Declaration of Helsinki was the principle for trial execution. The independent Medical Ethics Committee "Medisch Ethische Toetsingscommissie van de Stichting Beoordeling Ethiek Biomedisch Onderzoek" (Assen, the Netherlands) approved the study prior to any clinical study activity. All subjects provided written informed consent before participation. The trial was registered on ClinicalTrials.gov (NCT03161288).

SUBJECTS

Main inclusion criteria were male gender, 18 to 45 years of age with a body mass index between 18 and 30 kg/m^2 and previous immunization with TT more than 6 months prior to screening and no known previous exposure to KLH. Health status was verified by recording a detailed medical history,

a complete physical examination, vital signs, a 12-lead electrocardiogram (ECG) and laboratory testing (including hepatic and renal panels, complete blood count, virology and urinalysis). Subjects were excluded in case of any disease associated with immune system impairment, or use of prescription medication within two weeks prior to enrolment.

DOSE SELECTION AND REGIMEN

The starting dose of 0.006 mg/kg KY1005 was based on a minimal anticipated biological effect level (MABEL) principle using *in vitro* data obtained in human MLR experiments. The maximum dose of 12mg/kg KY1005 was based on the maximal effects observed in these experiments and predicted exposure equivalent to that at which maximum *in vivo* inhibition of the IgG response to KLH immunization occurred in monkeys. Detailed information on dose selection can be found in Supplementary Materials and Methods S1. The interval of four weeks between the loading dose and two maintenance doses was based on scaling of the KY1005 half-life (t½) of 23 ± 1 day observed in cynomolgus monkeys. Threefold dose increments between cohorts was based on a 30-fold difference in concentration between the lowest effects and maximal effects observed in the MLR experiments, a modest slope of the dose response and theoretical risks related to OX40L blocking.

STUDY DESIGN AND TREATMENTS

An overview of the study design is shown in Figure S1. Subjects were enrolled into eight cohorts. In each cohort, subjects were randomized to either 30 minutes of intravenous administration of KY1005 or placebo (6:2). Two subjects per cohort started as a sentinel group and if no safety issues arose within 48 hours after dosing, the remaining six subjects were dosed. The first three cohorts received single doses of 0.006 mg/kg, 0.018 mg/kg, or 0.05 mg/kg KY1005 respectively. The five subsequent cohorts received multiple doses starting with an initial loading dose of 0.15 mg/kg, 0.45 mg/kg, 1.35 mg/kg, 4 mg/kg, or 12 mg/kg KY1005 respectively followed by two maintenance doses of 50% of the loading dose, administered at four and eight weeks after the initial administration. Intramuscular KLH and TT immunizations were performed in the deltoid muscles one week after the last (third) KY1005/placebo dose in the MAD cohorts. KLH was administered in a formulation of 0.1 mg of subunit KLH (Immucothel®) adsorbed in 0.9 mg aluminium hydroxide (Alhydrogel®) into 0.5 mL NACL 0.9% as described previously.[30] TT was administered in the marketed formulation of ≥40 IU TT (Bilthoven Biologicals, Bilthoven, the Netherlands) in 0.5 mL NACL 0.9%.[31,32] Twenty-one days after intramuscular KLH administration, all subjects received an intradermal KLH administration in the left ventral forearm and placebo administration in the right ventral forearm. The formulation of 0.001 mg subunit KLH in 0.1 mL NACL 0.9% used for intradermal administration was based on a previously conducted trial.[30] The interval of twenty-one days between intramuscular KLH immunization and intradermal KLH administration and the interval of 48 hours between baseline and follow up skin challenge assessment has been used in previous other studies.[30,31,33–36] Prior to, and two days after the intradermal KLH administration, the skin challenge response was quantified.

SAFETY AND TOLERABILITY

Safety and tolerability were monitored by physical examination, assessment of vital signs, laboratory parameters (i.e., full blood count, biochemistry and urinalysis) and ECG data from 12-lead and 24-hour holter ECGS at regular intervals. Subjects were monitored continuously for adverse events (AES).

KY1005 PHARMACOKINETICS AND IMMUNOGENICITY

Serum concentrations of KY1005 for PK profiling and serum concentrations of anti-drug antibodies (ADAS) were measured by Eurofins Pharma Bioanalysis Services UK Ltd. (Abingdon, United Kingdom) using validated bioanalytical assay methods. The PK samples were analysed using a luminescent enzyme-linked immunosorbent assay (ELISA) with a lower limit of quantification (LLOQ) of 9.77 ng/mL. ADAS were measured using an electrochemiluminescence solid-phase extraction with acid dissociation method. In both ADA screening and confirmatory formats, the assay tolerated up to 100 µg/mL KY1005 at positive control anti-KY1005 antibody concentrations of 100 and 250 ng/mL.

OX40 AND OX40L EXPRESSION

OX40 and OX40L expression was measured by CHDR (Leiden, the Netherlands) on cell subsets of whole blood samples using flow cytometry. Red blood cell lysis was performed on heparinized whole blood using RBC lysis buffer (Thermo Fisher, Waltham, Massachusetts, USA). Leukocytes were stained with fluorochrome labelled antibodies at 4°C for 30 minutes, see Table S1

for a complete list. After staining, the cells were washed with PBS (Thermo Fisher). Samples were measured on a MACSQuant 10 analyser, and analysed using MACSQuantify software (both Miltenyi Biotec, Bergisch-Gladbach, Germany). See Figure S2 for the gating strategy. OX40 expression was assessed in $CD4^+$ and $CD8^+$ T cells, regulatory T cells and TH17 cells and OX40L expression was assessed in $CD19^+$ B cells and $CD14^+$ monocytes in all cohorts. In addition, expression of OX40 and OX40L in $CD4^+$ and $CD8^+$ effector memory and central memory cells was assessed in cohorts 4-8.

HUMORAL IMMUNITY TO KLH AND TT

The humoral response to intramuscular KLH and TT immunization was measured by anti-KLH and anti-TT IgM and IgG blood serum titres twenty-one days after immunization. Serum samples were assessed by quantitative ELISA for anti-KLH and anti-TT IgM and IgG levels as previously described.[30] In KLH-immunized subject blood samples, mean optical density (OD) of baseline samples was set to 1.00 and ratios relative to baseline were calculated for all subsequent samples. The LLOQ and the upper limit of quantification (ULOQ) for anti-KLH IgM and IgG were a baseline corrected OD of 0.060 and 3.900, respectively. The LLOQ and ULOQ for anti TT IgM were 10.0 IU/mL and 100 IU/mL, respectively and the LLOQ and ULOQ for anti-TT IgG were 0.100 IU/mL and 5.00 IU/mL, respectively.

CUTANEOUS BLOOD PERFUSION

Cutaneous blood perfusion quantification was performed with laser speckle contrast imaging (LSCI; PeriCam PSI System, Perimed AB, Järfälla, Sweden) as previously described.[30] In short, assessments were performed in a temperature controlled room (22°C) after acclimatization of the subjects. LSCI recordings of the target area on the left and right ventral forearms captured with the use of dedicated software (PimSoft, Perimed AB, Järfälla, Sweden). Circular regions of interest at the intradermal injection sites were defined and cutaneous blood perfusion (indicated as basal flow) was quantitatively assessed and expressed in arbitrary units (AUS). The homogeneity of cutaneous blood perfusion in the region of interest (indicated as flare), expressed as values that are +1 standard deviation (SD) from the mean basal flow within the region, was also quantitatively assessed and expressed in AUS. Illustrations of cutaneous blood perfusion measured with LSCI are depicted in Figure 1.

ERYTHEMA

Erythema quantification was performed with multispectral imaging (Antera 3D®, Miravex, Dublin, Ireland) as previously described.[30] In short, the camera was placed on the target area on the ventral forearms and images were captured using dedicated software (Antera 3D® software, Miravex, Dublin, Ireland). Circular regions of interest at the intradermal injection sites were defined and erythema was quantified using the average redness and CIELab a* Antera 3D® software modalities expressed as AUS. The average redness modality displays the distribution of redness using an internal software algorithm and the CIELab a* value, which is part of the CIELab colour space and expresses colour as a numerical value on a green–red colour scale.[37] Illustrations of erythema measured with multispectral imaging are depicted in Figure 1.

STATISTICS

Detailed statistical procedures used in the current study are provided in the Supplementary Materials and Methods S1. Subjects were randomized to KY1005 or placebo in a 3:1 ratio. Demographic and baseline variables were summarized by treatment. For safety and tolerability endpoints summary statistics for observed values were calculated for all continuous parameters. For every KY1005 dose, the peak serum concentration (C_{max}), $t_{1/2}$, area under the curve from zeropoint to the last measurable concentration ($AUC_{(0\text{-}last)}$) and clearance (CL) were reported as mean (coefficient of variation percentage (CV%)) and the time at which C_{max} is observed (T_{max}) was reported as median (range). PD endpoints measured at multiple time points post baseline were analysed with a mixed effect repeated measures model. Endpoints with one post-dose measurement were analysed with an analysis of covariance (ANCOVA) model. Skin challenge endpoints were analysed with ANCOVA with the change from the saline-injected control (right forearm) added as covariate. The general treatment effect and specific contrasts were reported with the estimated difference (ED), 95% confidence interval (CI) and p-value and graphically as ED, 95% CI and P-value or mean change from baseline (CFB), SD and P-value. Negative CFB values for skin challenge endpoints were possible due to measurement variability and the dynamic nature of the measurements. Non-linear mixed effects analysis of the exposure-response relationship was performed for anti-KLH and anti-TT antibody titres and skin challenge endpoints.

Results

BASELINE CHARACTERISTICS

The study was conducted between May 2017 and March 2018. Twenty-four (24) subjects were enrolled in the SAD part and forty (40) subjects in the MAD part of the study. Four (4) subjects did not complete the study: one (1) subject was withdrawn due to a suspected hypersensitivity reaction consisting of pruritus, swelling of the palate and gums and slurred speech lasting approximately 2 hours after the first dose, three (3) subjects withdrew consent for reasons unrelated to the study treatment. Baseline characteristics of all treatment groups are presented in Table 1.

SAFETY AND TOLERABILITY

No serious AEs (SAEs) occurred during the study. One (1) subject in the MAD part of the study (12mg/kg cohort) did not receive the second and third KY1005 doses due to a possible hypersensitivity reaction (mild palatal pruritus and swelling and slurred speech) after the first dose. No medication was administered based on the mild nature of the AEs and all symptoms resolved within two hours. Additional blood chemistry and haematology, including complement activation markers and tryptase were all within normal ranges. No other AE-related discontinuations occurred during the study. The most frequently occurring treatment emergent AE (TEAE) was headache (Table S2 and Table S3). All TEAEs were of mild (n=190) or moderate severity (n=16) and self-resolving without sequalae. Treatment did not result in any clinically significant changes in any safety laboratory parameters, physical examination, vital signs measures, 12-lead ECG recordings and holter ECG recordings (data not shown).

KY1005 PHARMACOKINETICS AND IMMUNOGENICITY

A dose-dependent increase in mean serum concentrations of KY1005 was observed after single administrations (cohorts 1-3) and after multiple administrations (cohorts 4-8) (Figure 2). KY1005 reached T_{max} approximately 4 hours after the start of infusion (median across cohorts from 0.5 - 24h) and had a t½ of approximately 24.3 days (mean across cohorts from 7.1 - 43.1 days with CV% of 15.5% - 51.8%) (Table 2). Overall, single or multiple doses of KY1005 as measured by noncompartmental PK analysis appeared to be nonlinear (Table 2). KY1005 clearance remained relatively stable at concentrations >2 mg/mL approximately (data not shown).

The number of subjects with ADAS increased with increasing KY1005 dose in cohorts 1 to 3 (two, two, and five subjects, respectively). In cohorts 4 to 8, however, the largest number of subjects positive for ADAS (four subjects) was observed at the lowest dose regime (0.15 mg/kg KY1005 cohort), with no subjects developing detectable ADAS at the highest dose (12 mg/kg KY1005 cohort). There was no correlation between ADAS and any of the PK parameters, including CL (data not shown).

OX40 AND OX40L EXPRESSION

No consistent OX40 and OX40L expression profile trends were observed across the groups, although some p-values <0.05 compared to placebo were noted (Table S4). The differences compared with placebo in OX40 and OX40L expression on a variety of immune cells did not induce any clinically relevant observations.

HUMORAL IMMUNITY TO KLH AND TT

Though no statistical significance was reached, KY1005 treatment seemed to suppress the anti-KLH IgM and IgG antibody response after intramuscular KLH immunization (Figure 3A and B). KY1005 appeared to have a PD effect from doses of 0.45 mg/kg and above based on the anti- KLH IgG response. The ED between KY1005 and placebo-treated subjects was maximally -32.4% (95% CI -54.7% - 0.9%, p=0.06) observed for anti-KLH IgG at the highest KY1005 dose of 12 mg/kg (Table 3). No consistent effect of KY1005 on anti-TT IgM and IgG antibodies was observed (Table 3 and Figure 3C and D).

EXPOSURE-RESPONSE MODELLING OF HUMORAL IMMUNITY TO KLH AND TT

Given the small sample size, post-hoc KY1005 exposure-response modelling was performed. This analysis showed a modest treatment effect of KY1005 (Akaike's Information Criteria (AIC)Emax model < AICno-effect model) on anti-KLH IgM (EMAX -0.22 AU, 95% CI -0.46 AU - 0.02 AU) and IgG antibody titres (EMAX -0.58 AU, 95% CI -1.10 AU– -0.06 AU), whereas no exposure-response was observed on anti-TT IgM and anti-TT IgG antibody titres (Figure 4A-D), based on the exposure-response model. The 50% of the maximal effect (EC_{50}) could not be reliably determined for any of the variables, likely due to the high variability of the measurements as well as the small sample size.

CUTANEOUS BLOOD PERFUSION

Overall, KY1005 reduced the intradermal KLH-driven increase in cutaneous blood perfusion quantified by LSCI basal flow and flare (Table 3 and Figure 3E and F). Although a clear dose dependence was absent, pharmacological KY1005 effects on LSCI basal flow and flare based on suppression of skin challenge response were observed at intermediate dose levels of 0.45 mg/kg (ED -13.4 AU, 95% CI -23.0 AU– -3.8 AU, p<0.01 and ED -7.5 AU, 95% CI -13.2 AU– -1.8 AU, p<0.05, respectively), 4 mg/kg (ED -11.0 AU, 95% CI -19.8 AU– -2.3 AU, p<0.05 and ED -5.8 AU, 95% CI -10.8 AU– -0.9 AU, p<0.05, respectively) and 12 mg/kg (ED -5.9 AU, 95% CI -14.4 AU - 2.5 AU, p=0.16 and ED -3.1 AU, 95% CI -7.9 AU–1.8 AU, p=0.21, respectively) (Figure 3E and F). All groups showed a reduced cutaneous blood perfusion response compared to placebo.

ERYTHEMA

KY1005 treatment also reduced erythema quantified by multispectral imaging as average redness and as CIELab a* (Table 3 and Figure 3G and H). Similar to the observations with LSCI multispectral imaging average redness and CIELab a* were decreased in the groups that received KY1005 as initial dose of 0.45 mg/kg (ED -0.20 AU, 95% CI -0.32 AU– -0.07 AU, p<0.01 and ED -2.1 AU, 95% CI -3.5 AU– -0.8 AU, p<0.01, respectively), 4 mg/kg (ED -0.17 AU, 95% CI -0.29 AU– -0.05 AU, P<0.01 and ED -2.0 AU, 95% CI -3.4 AU– -0.7 AU, p<0.01, respectively) and 12 mg/kg (ED -0.23 AU, 95% CI -0.35 AU– -0.11 AU, P<0.001 and ED -2.6 AU, 95% CI -4.0 AU– -1.3 AU, P<0.001, respectively) compared to placebo (Table 3 and Figure 3G and H).

EXPOSURE-RESPONSE MODELLING OF SKIN CHALLENGE ENDPOINTS

Exposure-response modelling showed a treatment effect of KY1005 ($AIC_{Emax\ model} < AIC_{no\text{-}effect\ model}$) on LSCI basal flow (EMAX -7.09 AU, 95% CI -13.23 AU– -0.96 AU), LSCI flare (EMAX -4.77 AU, 95% CI -8.06 AU– -1.48 AU), multispectral imaging average redness (Emax -0.20 AU, 95% CI -0.29 AU– -0.11 AU) and CIELab a* (EMAX -2.10 AU, 95% CI -3.05 AU– -1.15 AU) (Figure 4E-H), based on the exposure-response model. The EC50 could not be reliably determined for any of the variables, likely due to the high variability of the measurements as well as the small sample size.

Discussion

In this first-in-human study we showed that KY1005 was safe and well-tolerated and we demonstrated proof-of-pharmacology for KY1005 as the drug suppressed the KLH-driven neoantigen immune response via OX40-OX40L signalling interference, despite the observed variability in the skin challenge response.

Importantly KY1005 treatment in the current study had an unremarkable safety and tolerability profile. One hypersensitivity reaction was observed in this study in one subject in the 12 mg/kg group that was possibly related to KY1005. An allergy to KY1005 or any excipients was considered unlikely, since this was the first KY1005 exposure and subject had never received an intravenous administration of any kind. A pseudoallergy might have been the cause of the AEs. This pseudoallergy was classified as grade 1, since no medication was administered and the symptoms resolved spontaneously within two hours. No other AE-related discontinuations of KY1005 treatment occurred. AEs observed after monoclonal antibody administration are usually related to infection and immunomodulation.[38] No increase in infection rate after KY1005 treatment was observed compared to placebo, possibly explained by the fact that the OX40-OX40L pathway is primarily involved in sustaining T cell activation and not in the initial stimulation.[7]

PK analyses displayed a non-linear increase in mean serum concentrations of KY1005. The PK profile of KY1005 displayed non-linear target-mediated drug disposition (TMDD)[39] as is commonly observed for monoclonal antibodies.[40] At low KY1005 concentrations a high CL was observed as a large portion of the drug is likely cleared via drug-target binding and subsequent degradation of the drug-target complex. Saturation of TMDD presumably led to lower observed CL at higher KY1005 doses. The mean KY1005 t½ of 24.3 days was similar to the expected predicted mean t½ of 26 ± 7 days based on preclinical experiments. ADAs may influence the clearance of monoclonal antibodies. We did not find evidence for ADA-mediated clearance of KY1005, which may reflect no such effect, or an insufficient number of subjects exposed. At approximate concentrations of >2 mg/mL KY1005 clearance remained relatively stable which might indicate TMDD saturation and possibly 100% target binding.

Between 25% and 50% of KY1005 treated participants had positive ADA responses, but this was not associated with unexpected changes in the serum PK indicating where present the ADA were non or only weakly neutralising. The number of subjects with ADAs increased with increasing KY1005 dose in SAD cohorts. In MAD cohorts however, the largest number of subjects positive for ADAs was observed at the lowest dose regimen (0.15 mg/kg), with no subjects developing detectable ADAs at the highest dose regimen (12 mg/kg). This observation is in keeping with the pattern expected with increasing suppression of ADA development at higher doses reaching saturation of the target and suppression of antibody response to KY1005.

No statistically significant reduced antibody titres against KLH and TT were observed after KY1005 treatment compared to placebo. The lack of effect and consistency by dose group most possibly reflects the small sample size and normal variability observed with respect to the PD markers. Despite this the observed data indicated moderate pharmacological activity of KY1005 at loading doses of 0.45 mg/kg and above. Importantly, combined individual data of all KY1005 serum concentrations plotted against anti-KLH IgM and IgG antibody titres revealed a modest treatment effect of KY1005. This treatment effect was stronger on anti-KLH IgG compared to IgM possibly explained by the time window of 21 days between baseline and post-immunization measurements and class switching between the isotypes. Our results are translationally confirmed by a previously published study performed in mice which showed that blockade of the OX40-OX40L signalling pathway inhibited T cell-dependent antibody production after KLH immunization.[41] Based on these results, inhibition of OX40L may possibly interfere with T cell-dependent antibody production. Furthermore, maximum effects of KY1005 on anti-KLH antibody titres seem to have been reached based on the concentration-effect models. The recall antigen response to TT is probably not sufficiently suppressed as memory B cells are able to differentiate to plasma cells in the absence of T cells[42] and other pathways besides OX40-OX40L can still be stimulated. Although it is known that T cell-dependent B cell activation requires CD40-CD40L co-stimulatory factors following T cell receptor-Major Histocompatibility Complex II-peptide binding,[43,44] the exact mechanism and pathways underlying T cell-dependent B cell activation and the role of OX40-OX40L signalling remain to be elucidated. *Ex vivo* antigen re-challenges of lymphocytes isolated from KY1005-exposed volunteers may provide additional insight and improved characterization of immune pathways modulated by OX40-OX40L inhibition.

The KLH skin challenge model used in the present study was previously validated in healthy volunteers using multispectral imaging and LSCI similar to the methods used in the current study.[30] Various clinical studies have demonstrated that KLH is a potent immunostimulatory antigen, producing a robust immune response and having an excellent safety profile.[45] Formally, the study was not powered for detection of KY1005 effects on the skin challenge response;[30] the sample size used is common in first-in-human trials and the KLH-based pharmacodynamic skin challenge endpoints were exploratory in nature only. Despite being underpowered we found that KY1005 suppressed the skin challenge response following intradermal KLH administration as cutaneous blood perfusion and erythema were lower in KY1005-treated subjects compared to placebo. The half maximal inhibitory concentration (IC50) of KY1005 in preclinical *in vitro* experiments was 0.30 nM ± 0.01 nM (mean ± standard error of the mean). The KY1005 starting dose of 0.006 mg/kg corresponds to a concentration of 1.0 nM and was based on the MABEL principle using *in vitro* data obtained in human MLR experiments. Substantial decreases in cutaneous blood perfusion and erythema as a result of skin challenge response were initially observed at a KY1005 dose of 0.45 mg/kg which corresponds to a concentration of 75 nM, a 250-fold higher dose compared to the IC50. In contrast to KY1005's effects on anti-KLH antibodies, the effects on the skin challenge response were seen to be dose-dependent. Accordingly, exposure-response analyses displayed a treatment effect of KY1005 on all skin challenge endpoints (LSCI basal flow and flare and multispectral imaging average redness and CIELab a* values).

T cell-dependent immune responses are complex to monitor and to modulate. Therefore, a successful translation of the observed PD effects of KY1005 (suppression of the KLH-driven responses in healthy volunteers) to clinical effects in patients with immune mediated diseases is challenging. However, the unremarkable safety and tolerability profile of KY1005 combined with the observed immunomodulatory properties support the potential of KY1005 as a novel compound targeting the OX40-OX40L signalling pathway for immune mediated disorders. Based on the data generated in the present study, a successful Phase 2a trial of KY1005 has recently been completed in patients with atopic dermatitis and a Phase 2b is planned.

Table 1 Baseline characteristics.

	SAD				MAD					
	KY1005			Placebo	KY1005					Placebo
Loading dose	0.006 mg/kg	0.018 mg/kg	0.05 mg/kg	NA	0.15 mg/kg	0.45 mg/kg	1.35 mg/kg	4 mg/kg	12 mg/kg	NA
Maintenance doses	NA	NA	NA	NA	0.075 mg/kg	0.225 mg/kg	0.675 mg/kg	2 mg/kg	6 mg/kg	NA
	N=6	N=6	N=6	N=6	N=6	N=6	N=6	N=6	N=6	N=10
DEMOGRPAHICS										
Age (years)	24.3 (4.5)	23.8 (1.5)	25.3 (3.7)	26.2 (7.1)	28.0 (9.2)	23.3 (3.9)	24.8 (3.9)	24.7 (4.1)	34.2 (6.3)	28.5 (5.6)
BMI (kg/m^2)	22.9 (1.3)	21.7 (1.5)	23.5 (2.8)	23.5 (2.2)	22.9 (2.2)	23.1 (3.1)	22.8 (2.3)	23.1 (1.8)	24.9 (3.0)	24.5 (3.1)
VITAL SIGNS										
Systolic blood pressure (mmHG)	119 (8)	115 (11)	126 (11)	121 (16)	118 (3)	120 (11)	123 (13)	118 (11)	127 (7)	122 (9)
Diastolic blood pressure (mmHG)	72 (5)	68 (8)	77 (6)	68 (12)	72 (9)	75 (10)	68 (9)	70 (13)	78 (8)	72 (13)
Heart rate (BPM)	57 (13)	59 (7)	68 (11)	63 (17)	57 (8)	59 (8)	63 (14)	59 (10)	67 (9)	61 (8)
Temperature (°C)	36.7 (0.3)	36.9 (0.1)	36.8 (0.6)	36.7 (0.3)	36.4 (0.3)	36.9 (0.2)	36.5 (0.4)	36.5 (0.2)	36.6 (0.5)	36.6 (0.2)
LABORATORY TESTS										
Leucocytes ($*10^9$/L)	6.61 (1.05)	6.10 (1.58)	5.89 (1.06)	5.94 (0.96)	5.21 (1.16)	5.66 (0.86)	6.75 (1.52)	6.59 (1.59)	6.57 (1.19)	6.05 (2.42)
Thrombocytes ($*10^9$/L)	244.5 (26.4)	279.2 (68.1)	267.3 (43.0)	215.3 (40.1)	254.4 (69.8)	270.7 (53.8)	229.2 (29.6)	216.3 (53.3)	269.6 (65.7)	253.2 (53.7)
ALT (IU/L)	18.0 (5.8)	13.7 (4.2)	16.5 (3.6)	16.2 (7.1)	23.2 (14.1)	20.2 (11.7)	31.3 (20.1)	24.8 (19.0)	30.3 (11.7)	22.2 (7.0)
AST (IU/L)	23.2 (9.1)	20.7 (5.3)	19.2 (3.6)	18.2 (5.1)	22.7 (6.0)	24.0 (4.8)	28.5 (9.4)	25.3 (8.7)	27.0 (8.7)	21.2 (5.8)

Parameters are shown as mean (standard deviation). SAD=single ascending dose, MAD=multiple ascending dose, NA=not applicable.

Table 2 Summary of pharmacokinetic parameters of KY1005 per dose level.

Parameter	SAD			MAD				
1st KY1005 dose	0.006 mg/kg (N=6)	0.018 mg/kg (N=6)	0.05 mg/kg (N=6)	0.15 mg/kg (N=6)	0.45 mg/kg (N=6)	1.35 mg/kg (N=6)	4 mg/kg (N=6)	12 mg/kg (N=6)
T_{max} (hr)	0.5 (0.5-4.0)	4.0 (0.5-12.0)	2.3 (0.5-4.0)	0.5 (0.5-4.0)	4.0 (0.5-12.0)	2.3 (0.5-24.0)	2.3 (0.5-4.5)	4.0 (0.5-24.0)
C_{max} (µg/mL)	0.1 (16.2%)	0.4 (40.4%)	1.3 (6.7%)	4.0 (12.4%)	11.8 (10.3%)	34.8 (24.0%)	112.2 (33.8%)	289.7 (17.5%)
t½ (days)	7.1 (33.3%)	13.4 (41.3%)	12.1 (18.6%)	20.8 (26.9%)	23.1 (26.2%)	23.2 (44.7%)	20.3 (17.9%)	22.7 (21.9%)
$AUC_{(0\text{-}last)}$ (µg*day/mL)	0.9 (33.9%)	3.1 (31.1%)	17.3 (15.4%)	43.0 (8.9%)	138.5 (11.5%)	453.3 (18.2%)	1263.7 (30.3%)	3337.7 (7.5%)
CL (mL/min)	0.30 (33.2%)	0.27 (58.7%)	0.15 (15.5%)	0.11 (19.7%)	0.10 (19.5%)	0.10 (26.5%)	0.12 (25.8%)	0.12 (19.1%)
2nd KY1005 dose	NA	NA	NA	0.075 mg/kg (N=6)	0.225 mg/kg (N=5)	0.675 mg/kg (N=6)	2 mg/kg (N=6)	6 mg/kg (N=5)
T_{max} (hr)	-	-	-	2.5 (0.5-12.5)	24.0 (4.6-24.0)	2.5 (0.5-24.0)	12.5 (0.5-24.0)	4.6 (0.5-24.0)
C_{max} (µg/mL)	-	-	-	2.9 (20.9%)	9.8 (21.3%)	26.1 (19.3%)	89.6 (31.1%)	229.9 (11.7%)
t½ (days)	-	-	-	27.0 (51.8%)	30.1 (NA)	25.1 (18.3%)	23.4 (32.0%)	24.7 (15.5%)
$AUC_{(0\text{-}last)}$ (µg*day/mL)	-	-	-	41.2 (12.2%)	157.6 (12.5%)	380.1 (18.6%)	1314.5 (22.4%)	3833.9 (10.8%)
CL (mL/min)				0.11 (27.9%)	0.08 (NA)	0.10 (2.2%)	0.09 (11.6%)	0.11 (21.4%)
3rd KY1005 dose	NA	NA	NA	0.075 mg/kg (N=6)	0.225 mg/kg (N=5)	0.675 mg/kg (N=6)	2 mg/kg (N=5)	6 mg/kg (N=5)
T_{max} (hr)	-	-	-	4.5 (0.5-12.5)	4.5 (4.5-24.0)	4.5 (0.5-12.5)	24.0 (24.0-24.0)	0.5 (0.5-24.0)
C_{max} (µg/mL)	-	-	-	3.0 (15.7%)	11.5 (16.6%)	28.8 (18.3%)	73.8 (9.9%)	249.6 (14.4%)
$T_{1/2}$ (days)	-	-	-	27.7 (20.5%)	28.3 (24.6%)	43.1 (24.1%)	41.7 (25.9%)	24.3 (17.3%)
$AUC_{(0\text{-}last)}$ (µg*day/mL)	-	-	-	53.0 (15.2%)	195.5 (9.9%)	551.9 (8.1%)	1674.8 (10.6%)	4505.2 (9.7%)
CL (mL/min)				0.09 (20.3%)	0.07 (21.0%)	0.06 (13.1%)	0.06 (22.9%)	0.10 (35.7%)

Data displayed as mean (coefficient of variation %) and for T_{max} as median (range). SAD=single ascending dose, MAD=multiple ascending dose, C_{max}=peak serum concentration, T_{max}=time at which the C_{max} is observed, t½=half-life, $AUC_{(0\text{-}last)}$=area under the curve from zeropoint to the last measurable concentration, CL=clearance, NA=no regression line could be fitted.

Table 3 Summary statistics for pharmacodynamic endpoints.

Pharmaco-dynamic parameter	KY1005 dose level				
	0.15 mg/kg (N=6)	0.45 mg/kg (N=5)	1.35 mg/kg (N=6)	4 mg/kg (N=5)	12 mg/kg (N=5)
Anti-KLH IGM (% change)	-0.1% (-14.9%–17.4%)	-3.1% (-18.3%–14.9%)	-9.2% (-22.7%–6.6%)	0.7% (-15.1%–19.4%)	-7.1% (-21.7%–10.1%)
Anti-KLH IGG (% change)	-4.8% (-34.8%–39.0%)	-23.9% (-49.0%–13.5%)	-13.9% (-41.0%–25.7%)	-14.0% (-42.4%–28.3%)	-32.4% (-54.7%–0.9%)
Anti-TT IGM (% change)	-2.6% (-18.4%–16.2%)	-3.6% (-20.0%–16.1%)	-6.7% (-22.6%–12.5%)	2.7% (-15.1%–24.1%)	-3.2% (-20.0%–17.2%)
Anti-TT IGG (% change)	13.8% (-19.6%–61.2%)	11.0% (-23.2%–60.5%)	3.6% (-26.1%–45.2%)	2.4% (-28.6%–46.9%)	4.6% (-27.0%–49.9%)
LSCI basal flow (AU)	-3.3 (-11.3–4.7)	-13.4 (-23.0–-3.8)**	-6.2 (-14.2–1.8)	-11.0 (-19.8–-2.3) *	-5.9 (-14.4–2.5)
LSCI flare (AU)	-2.2 (-6.9–2.4)	-7.5 (-13.2–-1.8)*	-4.5 (-9.0–0.1)	-5.8 (-10.8–-0.9) *	-3.1 (-7.9–1.8)
Multispectral imaging average redness (AU)	-0.04 (-0.16–0.09)	-0.20 (-0.32–-0.07)**	-0.11 (-0.23–0.01)	-0.17 (-0.29–-0.05) **	-0.23 (-0.35–-0.11) ***
Multispectral imaging CIELab a* (AU)	-0.7 (-2.1–0.6)	-2.1 (-3.5–-0.8)**	-1.2 (-2.5–0.1)	-2.0 (-3.4–-0.7)**	-2.6 (-4.0–-1.3) ***

Data displayed as estimated difference (95% confidence interval). AU=arbitrary unit.
** P<0.05, ** P<0.01, *** P<0.001.*

Figure 1 Illustrations of LSCI basal flow and erythema assessed as average redness with multispectral imaging. Images were taken at intradermal KLH injection site 2 days after intradermal KLH administration of a subject treated with an initial KY1005 12 mg/kg dose (left images) and a subject that received placebo (right images).

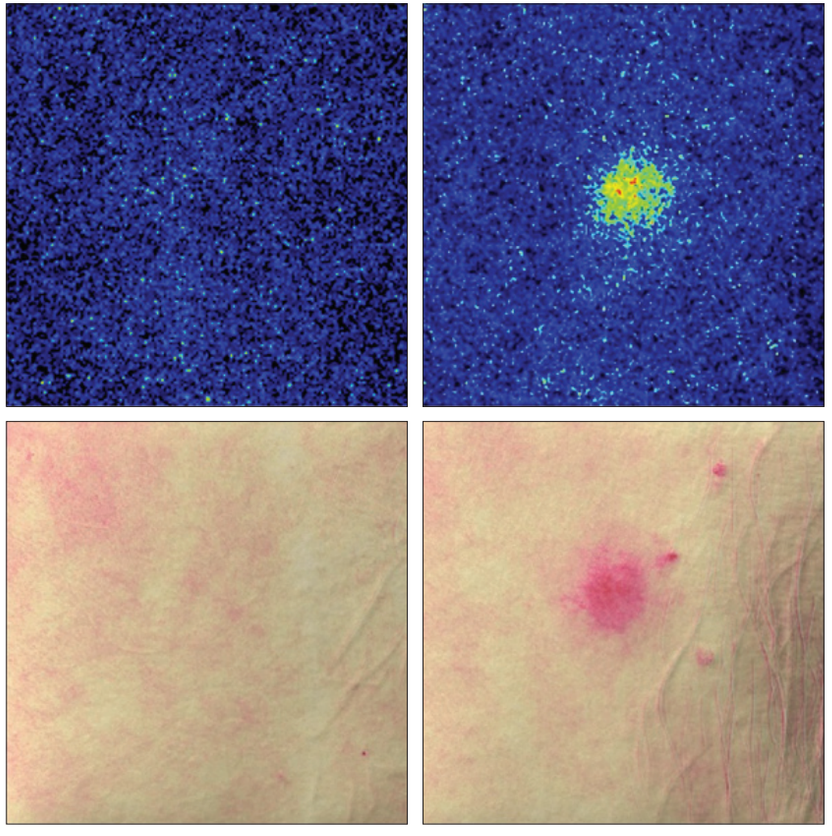
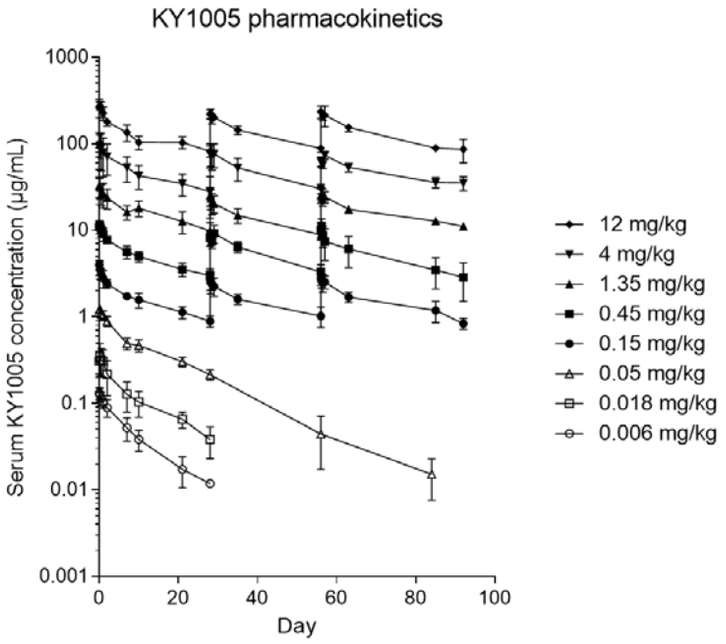


Figure 2 KY1005 serum concentrations (µg/mL). Data displayed on log10 scale as mean (standard deviation).



All mentioned supplementary figures and tables in this chapter can be found on the corresponding website by scanning this QR code.

Figure 3 Anti-KLH IgM (A) and IgG antibody titres (B), anti-TT IgM (C) and IgG antibody titres (D) 21 days after KLH immunization, cutaneous blood perfusion by LSCI basal flow (E) and LSCI flare (F), erythema by multispectral imaging average redness (G) and multispectral imaging CIELab a* (H) 2 days after intradermal KLH administration by treatment group. Data are shown as estimated difference percentage change (95% confidence interval) for A-D and as mean change from baseline (standard deviation) for E-H. ED=estimated difference, AU=arbitrary unit, CFB=change from baseline, i.d.=intradermal, KLH=keyhole limpet haemocyanin. P-values are based on estimated differences between groups with correction for baseline measurements and saline administration. *p<0.05, **p<0.01, ***p<0.001.

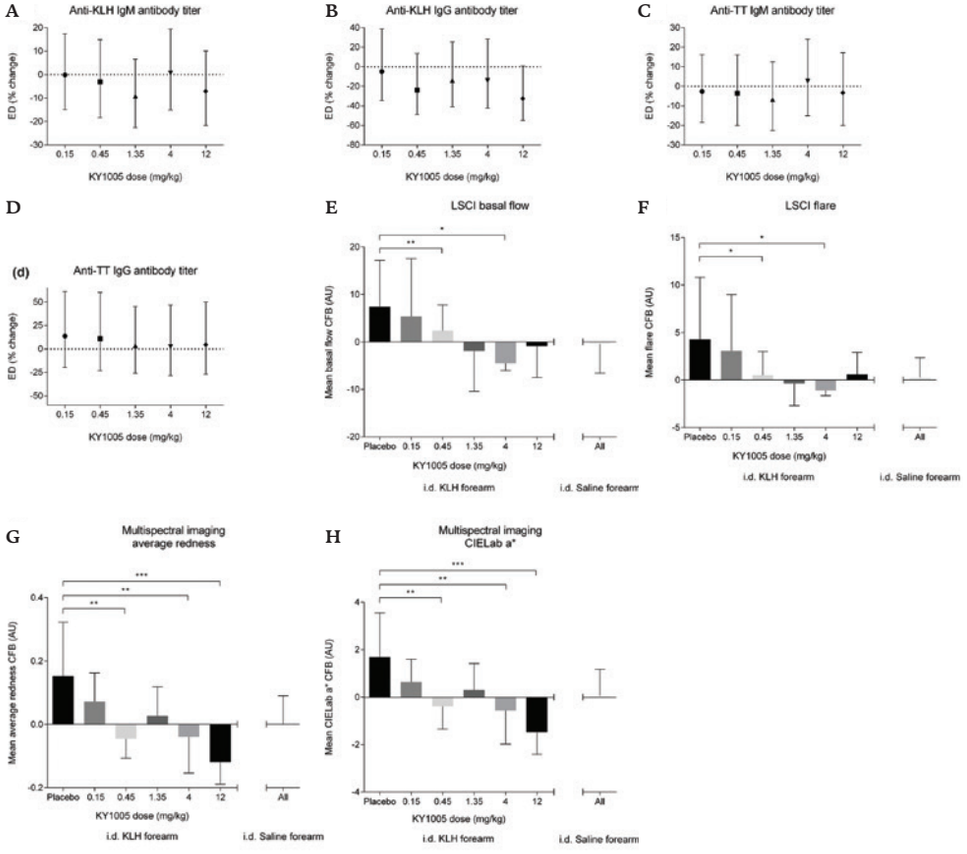
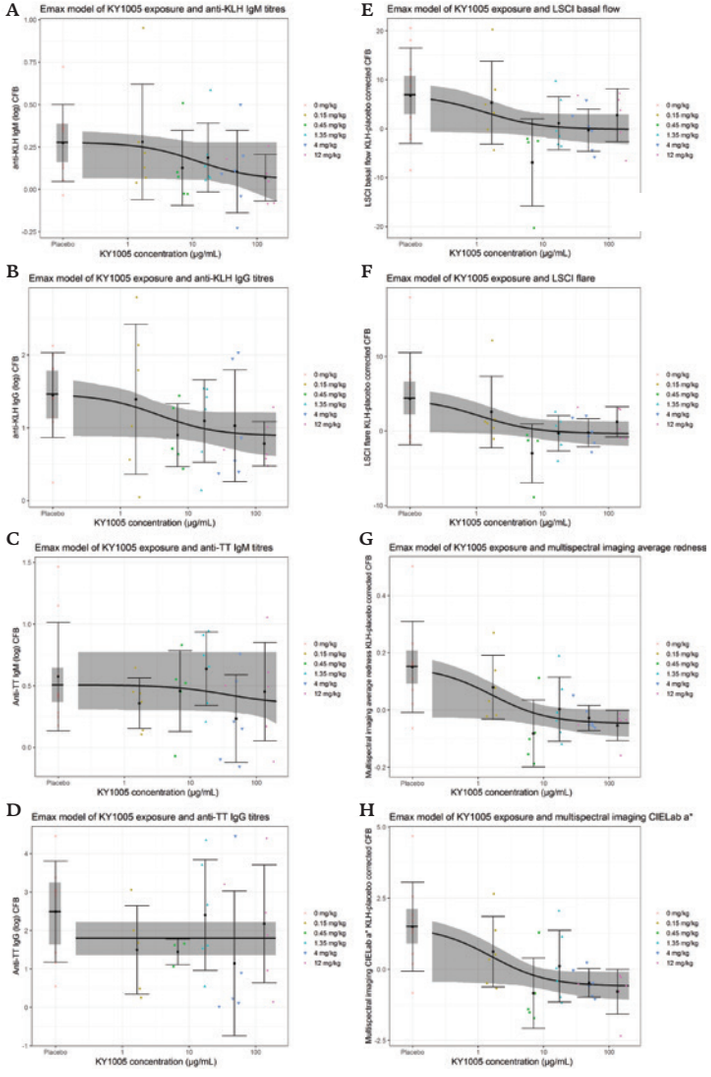


Figure 4 EMAX model of KY1005 exposure and anti-KLH IgM (A), anti-KLH IgG (B), anti-TT IgM (C), anti-TT IgG (D), LSCI basal flow (E), LSCI flare (F), multispectral imaging average redness (G) and multispectral imaging CIELab a* (H). Dots represent individual data points. Black squares (error bars) represent mean (standard deviation) of observed data per dose level. Black line (grey area) represents model predicted mean (90% confidence interval). Data are shown as log10 change from baseline ratios vs.



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CHAPTER 9

Stimulation of the PD-1 pathway decreases atherosclerotic lesion development in Ldlr deficient mice

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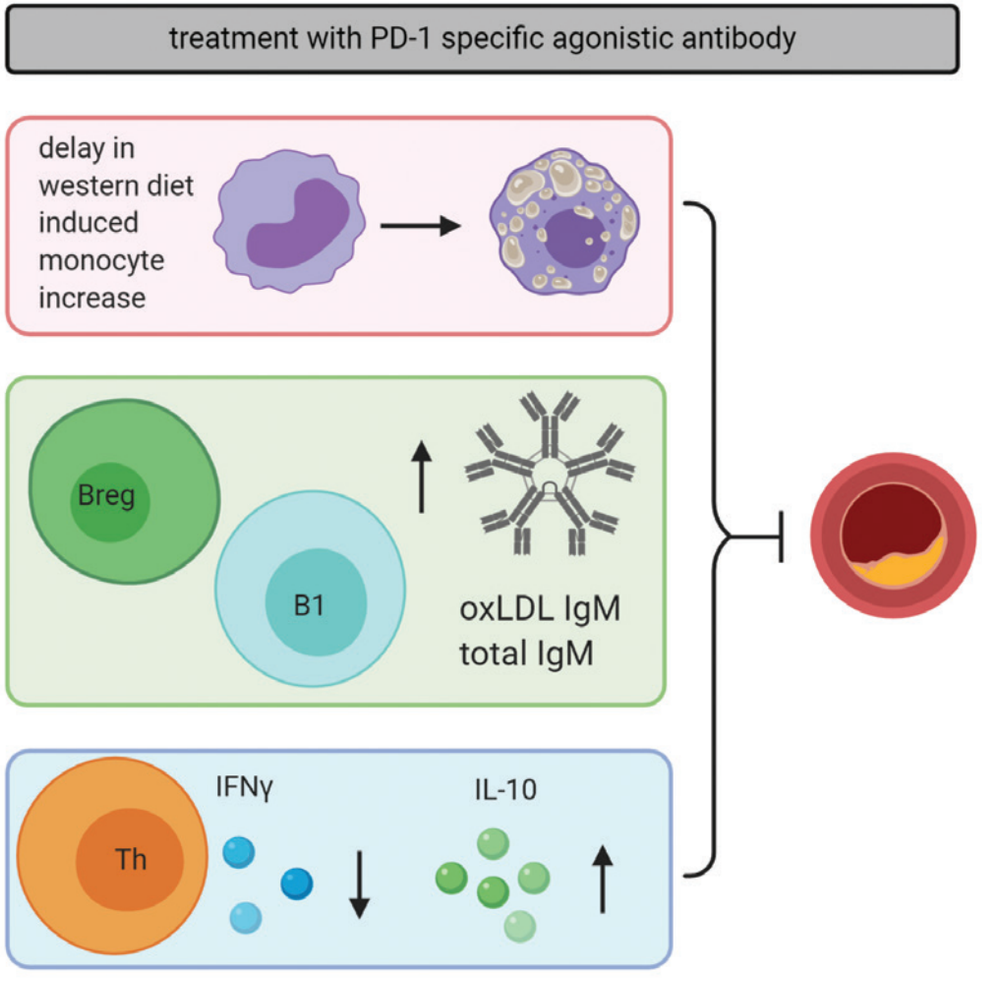
Graphical abstract



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Abstract

AIM Signaling through the coinhibitory programmed death (PD)-1/PD-L1 pathway regulates T cell responses and can inhibit ongoing immune responses. Inflammation is a key process in the development of atherosclerosis, the underlying cause for the majority of cardiovascular diseases.Dampening the excessive immune response that occurs during atherosclerosis progression by promoting PD-1/PD-L1 signaling may have a high therapeutic potential to limit disease burden. In this study we therefore aimed to assess whether an agonistic PD-1 antibody can diminish atherosclerosis development.

METHODS AND RESULTS LDLR$^{-/-}$ mice were fed a western-type diet (WTD) while receiving 100μg of an agonistic PD-1 antibody or control vehicle twice a week. Stimulation of the PD-1 pathway delayed the WTD-induced monocyte increase in the circulation up to 3 weeks and reduced T cell activation and proliferation. CD4^{+} T cell numbers in the atherosclerotic plaque were reduced upon PD-1 treatment. More specifically, we observed a 23% decrease in atherogenic IFNγ-producing splenic CD4^{+} T cells and a 20% decrease in cytotoxic CD8^{+} T cells, whereas atheroprotective IL-10 producing CD4^{+} T cells were increased with 47%. Furthermore, we found an increase in regulatory B cells, B1 cells and associated atheroprotective circulating OXLDL-specific IgM levels in agonistic PD-1-treated mice. This dampened immune activation following agonistic PD-1 treatment resulted in reduced atherosclerosis development ($p<0.05$).

CONCLUSIONS Our data show that stimulation of the coinhibitory PD-1 pathway inhibits atherosclerosis development by modulation of T- and B cell responses. These data support stimulation of coinhibitory pathways as a potential therapeutic strategy to combat atherosclerosis.

Introduction

Atherosclerosis is a chronic autoimmune disease characterized by the accumulation of lipids and immune cells, such as macrophages and pro-atherogenic IFNγ-producing TH1 cells, in the atherosclerotic plaque.[1,2] During disease progression, immune cells respond to atherosclerosis-specific antigens, such as APOB100, the primary protein in low-density lipoprotein (LDL), which are presented via MHC molecules on the surface of antigen-presenting cells (APCs). Subsequent activation of immune cells is regulated by a network of costimulatory and coinhibitory molecules present on both T cells and APCs. The most familiar costimulatory network is the B7/CD28 family, which has proven to be detrimental for TH1-driven atherosclerosis.[3] In the past two decades, interference in other costimulatory networks, including the CD40-CD40L and OX40-OX40L pathways, confirmed its potential to inhibit experimental atherosclerosis and to target a broad range of immune responses involved in this disease process.[4,5] Whereas costimulatory molecules need to be suppressed to dampen the overactive immune system in atherosclerosis, signaling through coinhibitory pathways must be promoted. The interaction of programmed death (PD)-1 with PD-L1/2 forms such a coinhibitory pathway, and can inhibit proliferation, cytokine production, cytolytic function, and survival of T cells.[6] PD-1 expression is not restricted to activated T cells,[7,8] but can also be upregulated on B cells and certain dendritic cells upon antigen stimulation.[8,9] PD-L1 is expressed by a large variety of cell types, including T cells, macrophages, dendritic cells and endothelial cells.[10-12] Previously it has been shown that the PD-1/PD-L1 pathway is a key regulator of many autoimmune diseases, including rheumatoid arthritis,[13] multiple sclerosis,[14] and cardiac inflammation.[15]

In cardiovascular disease patients, altered levels of circulating PD-1 and PD-L1 expressing cells have been reported.[16,17] For example, PD-1 expression on circulating T cells was decreased compared to healthy control individuals and concomitantly, decreased PD-L1 expression on APCs was reported, which corresponded to increased T cell responses.[18] Recent proteomics analysis of human atherosclerotic plaques revealed the presence of PD-1 expressing T cell populations inside the advanced atherosclerotic plaque as well.[19] Moreover, PD-1/PD-L1 deficiency aggravates experimental atherosclerosis in LDL receptor deficient (LDLR$^{-/-}$) mice,[20-22] with increased numbers of pro-atherogenic CD4^{+} and CD8^{+} T cells in the plaque.

It is however unknown whether stimulation of PD-1 signaling can inhibit atherosclerosis. Previous studies have shown that stimulation of coinhibitory molecules, such as CTLA-4 and BTLA, suppressed pro-atherogenic T- and B cell immunity and decreased atherosclerosis development in APOE$^{-/-}$ and LDLR$^{-/-}$ mice, respectively.[23,24] In addition, Seko et al showed that treatment of C3H/HE mice with an agonistic PD-1 antibody protected against virus-induced myocarditis.[25]

Together, these findings fuel the hypothesis that stimulation of the PD-1 pathway can limit the overactive immune system during atherosclerosis development and thus inhibit plaque progression. Therefore, LDLR$^{-/-}$ mice fed a western-type diet (WTD) were treated with a stimulatory PD-1 antibody for either 2 or 6 weeks to determine the effects on atherosclerosis development and the atherosclerosis-related immune response.

Methods

ANIMALS

LDLR$^{-/-}$ mice on a C57BL/6J background were purchased from Jackson Laboratory (Sacramento, CA, USA) and bred in-house. Animals were kept under standard laboratory conditions; food and water were provided ad libitum. In order to develop atherosclerotic lesions, female mice (8-12 weeks old) were fed a western-type diet (WTD, 0.25% cholesterol, 15% cocoa butter (SDS, Essex, UK)) for 2 or 6 weeks. The agonistic PD-1 antibody[25] (clone: PIM-2, 100 µg/mouse), isotype control (Tebu-Bio, Heerhugowaard, The Netherlands) or PBS were injected intravenously twice a week in 100µl volumes. Mice were randomized over the groups using baseline age, weight and cholesterol levels. During the experiments, blood samples were obtained by tail vein bleeding. At the end of experiments, mice were anaesthetized by a subcutaneous injection of a cocktail containing ketamine (40 mg/ml), atropine (0.1 mg/ml), and xylazine (8 mg/ml). Mice were bled followed by perfusion with phosphate-buffered saline (PBS) through the left cardiac ventricle. Total white blood cell count and monocyte content in blood were analyzed using an automated XT-2000iV veterinary hematology analyzer (Sysmex Europe GMBH, Norderstedt, Germany). All animal work was performed in compliance with the Dutch government guidelines and the Directive 2010/63/EU of the European Parliament. Experiments were approved by the Ethics Committee for Animal Experiments of Leiden University.

CELL PREPARATION

Upon sacrifice, K_2EDTA anti-coagulated blood, serum, spleens and hearts were harvested. Single-cell suspensions of spleens were obtained using a 70-µm cell strainer (Greiner Bio-one, Kremsmunster, Austria). WBCS were obtained by lysing the blood and splenocytes with ACK lysis buffer (0.15 M NH_4CL, 1 mM $KHCO_3$, 0.1 mM NA_2EDTA, pH 7.3). Hearts were embedded in OCT compound (Sakura, Tokyo, Japan) and stored at -80°C until further processing.

FLOW CYTOMETRY

Cell suspensions were stained using fluorochrome-labeled antibodies. A complete antibody list is provided in Table S1. Intracellular cytokine staining was performed after stimulation with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) and 200 ng/mL ionomycin (both Sigma-Aldrich, Deisenhofen, Germany) for 4 hours in the presence of brefeldin A (Thermo Fisher Scientific, Waltham, MA, USA). Samples were fixed and permeabilized using the fixation and permeabilization kit (BD Biosciences) prior to intracellular staining. Flow cytometry analyses were performed on a Cytoflex S (Beckman Coulter, Brea, CA, USA) or MACSQuant 16 analyzer (Miltenyi Biotec, Bergisch Gladbach, Germany) and FlowJo software (Treestar, San Carlos, CA, USA) or Flowlogics software (Inivai, Mentone, Australia).

IMMUNOHISTOCHEMISTRY

To determine plaque size, 10 µm sections of the aortic root were prepared and collected. Mean plaque size was calculated using 5 sequential sections stained with Oil-Red-O (ORO) and hematoxylin (both Sigma Aldrich). Intraplaque collagen and necrotic core content were quantified after staining with Masson's Trichrome staining kit (Sigma Aldrich) according to the manufacturer's protocol. Corresponding sections on separate slides were stained for monocyte/macrophage content with a MOMA-2 antibody (1:1000, rat IgG2B, Serotec Ltd.) followed by a goat anti-rat IgG-alkaline phosphatase antibody (1:100, Sigma-Aldrich). $CD4^+$ and $CD8^+$ T cells were stained using CD4 (RM4-5, 1:90, ThermoFisher) and CD8A (LY-2, 1:100, eBioscience) antibodies, and a secondary rabbit anti-rat IgG antibody (BA-4001, Vector), followed by the Vectastain ABC kit (PK-4000, Vector). Color development was achieved using NOVARED peroxidase (Vector laboratories) as enzyme substrate. T cells were scored manually. The relative amount of collagen and that of macrophages in the lesions is expressed as the percentage collagen- or MOMA-2-positive area of the total lesion surface area. Spleens were sectioned at a 10 µm thickness and were stained with hematoxylin & eosin (Sigma Aldrich) for white pulp quantification and with ORO and hematoxylin to analyze lipid content. All morphometric analyses were performed in a blinded fashion on a Leica CTR6000B microscope with mikroCam II 20mp (Bresser) using Leica QWin software (Leica Imaging Systems, UK) or ImageJ (FIJI).

SERUM MEASUREMENTS

Total cholesterol levels were assessed in serum using an enzymatic colorimetric assay (Roche/Hitachi, Mannheim, Germany). Precipath (Roche/Hitachi) was used as internal standard. Total serum levels of IgM and OXLDL-specific IgM were determined by ELISA as described previously.[26]

PROLIFERATION ASSAY

Splenocytes were cultured in triplicate in a 96-wells round-bottom plate (2×10^5 cells/well) in RPMI1640 + 10% FCS + 100 U/ml streptomycin/penicillin. Cells were stimulated with anti-CD3 and anti-CD28 (2µg/ml) for 48 hours. Proliferation was measured by addition of 3H-thymidine (0.5 µCi/well, Amersham Biosciences, the Netherlands) for the last 16 hours. The amount of 3H-thymidine incorporation was measured using a liquid scintillation analyzer (Tri-Carb 2900R, Perkin Elmer). Responses are expressed as stimulation index (SI): ratio of mean counts per minute of triplicate cultures with stimulation to triplicate cultures without stimulation.

STATISTICAL ANALYSIS

Data are reported as mean ± standard error of mean (SEM). Differences between groups were calculated using a Student's *t*-test, or one way ANOVA with Dunnet's post hoc analysis when 3 groups were compared. When 3 groups were compared, the control chow group and PD-1 agonist group receiving WTD were both compared to the control WTD group. Statistical analyses were performed using Graphpad Prism version 8 (Graphpad, San Diego, CA, USA). One mouse in the control group did not develop any atherosclerosis and was therefore excluded as an outlier (ROUT method) from the data.

Results

PD-1 STIMULATION PROMOTES ANTI-ATHEROGENIC IMMUNITY

To assess the short-term effects of PD-1 stimulation on the immune system, LDLR$^{-/-}$ mice were treated for 2 weeks with a PD-1 agonist or control vehicle while receiving a WTD diet. Additionally, a control group was kept on chow diet to be able the assess the direct effects of the high cholesterol diet on the immune system (Figure 1A). As shown in Figure 1B, cholesterol levels increased upon WTD administration and no differences in body weight were observed between the experimental groups. Administration of a WTD significantly increased the relative amount of monocytes in peripheral blood (chow: 9.6 ± 1.1% vs. WTD: 15.4 ± 1%, P=0.004, Figure 1C), which was less pronounced in the PD-1 stimulated WTD-fed mice (12.4 ± 1.3%). Similar patterns were seen for both the patrolling (LY6C^{int}) and inflammatory monocyte (LY6C^{hi}) subsets, suggesting that agonistic PD-1 treatment did not affect a specific monocyte subset. Absolute total monocyte numbers as measured by automated hematology analysis revealed a similar trend, albeit these data did not reach significance (P=0.15, Figure S1). An additional experiment shows that the relative monocyte effect is PD-1 specific, since monocyte levels upon isotype control treatment (Figure S2A) correspond to those of the PBS treated mice (Figure 1C). In contrast, the percentage of splenic monocytes was increased in PD-1 stimulated mice (3.5 ± 0.3%) compared to control WTD (2.2 ± 0.2%, P=0.004) and control chow (0.6 ± 0.1%, Figure 1D). Despite enlarged spleens in mice treated with the PD-1 agonist (Figure S3A), we did not observe significant differences in T- or B cell percentages compared to the control vehicle treated mice (Figure S3B). As the PD-1/PD-L1 pathway inhibits proliferation of activated T cells,[6] the proliferative capacity of splenocytes isolated from either control or PD-1 stimulated mice was measured after stimulation with anti-CD3/CD28 antibodies. As shown in Figure 1E, PD-1 stimulation resulted in a 75% decrease in T cell proliferation compared to the controls (PD-1: 12.0 ± 6.8 S.I. vs. control: 47.8 ± 4.9 S.I., P=0.0003). PD-1 stimulation did not alter the percentages of CD4$^+$, CD8$^+$ or total CD19$^+$ in the periphery (Figure S3C). Interestingly, we did observe increased circulating regulatory B cells (BREGS), defined as CD19$^+$CD5$^+$CD1d^{hi} cells, upon 2 weeks of PD-1 stimulation (PD-1: 1.0% ± 0.1% vs control WTD: 0.6% ± 0.1%, P=0.02) (Figure 1F).

REDUCED T CELL ACTIVATION AND PRO-ATHEROGENIC IFNγ-PRODUCING T CELLS UPON AGONISTIC PD-1 TREATMENT

Since PD-1 stimulation promoted anti-atherogenic responses in our short-term experiment, we next investigated the immunomodulatory effect of PD-1 stimulation during atherosclerosis development. LDLR$^{-/-}$ mice were fed a WTD for 6 weeks while receiving an agonistic PD-1 antibody or control vehicle (Figure 2A). During the experiment we observed an increase in serum cholesterol in PD-1 stimulated mice, while no difference in weight was observed between the groups (Figure 2B). In line with our previous findings (Figure 1C), PD-1 stimulated mice show a decrease in circulating monocytes (2.4 ± 0.4%) compared to control mice (5.4 ± 0.5%, P=0.001, Figure 2C) after 3 weeks. At sacrifice, circulating monocyte percentages did not differ anymore between the groups. No differences in relative monocyte content was observed in the spleen after 6 weeks of treatment, except for a significant decrease in patrolling monocytes (PD-1: 1.6 ± 0.1% vs. control: 2.2 ± 0.2%, P=0.03) (Figure 2D). Again, spleen weight was increased in PD-1 stimulated mice, however no differences were found in the relative white pulp content of the spleen (PD-1: 21.6 ± 1.0% vs. control: 23.4 ± 1.0%) and no excess fat depositions were found in spleens of PD-1 agonistic treated mice compared to spleens of control mice (Figure S4A). Percentages and absolute values of total CD4$^+$ and CD8$^+$ T cells were not affected in the spleen (Figure 3A and Figure S4B). Similarly, circulating CD4$^+$ and CD8$^+$ T cells and their activation status was unchanged (Figure S5). However, the activation status of splenic T cells, as measured by the expression of activation marker CD69, was decreased in PD-1 agonist treated mice (PD-1: 23.3 ± 4.6% vs. control: 29.2 ± 5.4%, P=0.005) (Figure 3A). Moreover, a significant decrease in pro-atherogenic IFNγ-producing cells was seen in both CD4$^+$ and CD8$^+$ populations upon PD-1 stimulation (CD4$^+$ T cells: PD-1: 5.1 ± 0.9% vs. control: 6.6 ± 1.6% P=0.005; CD8$^+$ T cells: PD-1: 4.9 ± 0.8% vs. control: 6.2 ± 1.2% for control, P=0.006) (Figure 3B, C, Figure S6), whereas a significant increase in atheroprotective CD4$^+$ IL-10-producing cells was found after PD-1 stimulation (PD-1: 2.8 ± 0.8% vs. control: 1.9 ± 0.8%, P=0.003). In the lymph nodes draining from the heart, we did not observe differences in T cell numbers or activation status (data not shown). Locally in the atherosclerotic plaque, a decrease in the number of CD4$^+$ T cells after PD-1 stimulation was observed (PD-1: 4.5 ± 0.6 vs. control:

7.0 ± 0.8, p=0.008) (Figure 3D), while the number of CD8$^+$ T cells was relatively low and did not significantly differ between the groups (PD-1: 1.2 ± 0.4 vs. control: 0.8 ± 0.2, P=0.61) (Figure S5C).

INCREASED CIRCULATING B1 CELLS AND OXLDL-SPECIFIC IGM IN AGONISTIC PD-1 TREATED MICE

As T cells play an important role in B cell activation, and PD-1 is also expressed on activated B cells, we anticipated PD-1 stimulation may also affect humoral immunity during atherosclerosis development. Although total circulating B cells were unaltered after 6 weeks of PD-1 stimulation, we did observe a significant increase in B1 cells (PD-1: 6.9 ± 1.4% vs. control: 5.4 ± 1.7%, P=0.01, Figure 4A). In line with this finding, total serum IgM levels as well as OXLDL-specific IgM levels were increased by PD-1 stimulation (Figure 4B). Notably, the levels of OXLDL IgM were inceased to a greater extent than total IgM, indicating also a relative increase of atheroprotective IgM. Total OXLDL-specific IgG were also increased after PD-1 stimulation. Similarly to the short-term experiment, an increase of circulating BREGS was observed, although not statistically significant (PD-1: 0.34 ± 0.07% vs. control: 0.23 ± 0.05%, Figure 4A).

PD-1 STIMULATION REDUCES PLAQUE DEVELOPMENT IN THE AORTIC ROOT

Given the immunosuppressive effect of PD-1 stimulation, we investigated the effect of PD-1 stimulation on atherosclerotic plaque development. LDLR$^{-/-}$ mice treated with an agonistic PD-1 antibody showed a 26.4% smaller plaque size compared to the control group (PD-1: 2.40 ± 0.25 x 10^5 μm^2 vs. control: 3.22 ± 0.25 x 10^5 μm^2, P=0.04) (Figure 5A). Similarly, when plaque size was calculated as a percentage of the total lumen area a significant decrease was seen in PD-1 stimulated mice (PD-1: 23.0 ± 1.8% vs. control: 30.0 ± 2.1%, P=0.02). The percentage of collagen in the plaque was similar in both groups (PD-1: 27.3 ± 2.2% vs. control: 28.0 ± 1.5%, Figure 5B). Likewise, the necrotic core content of the plaque (PD-1: 14.1 ± 1.9% vs. control: 19.0 ± 2.2%) and the macrophage content in the plaque as assessed by MOMA-2 staining (PD-1: 30.5 ± 2.8% vs. control: 32.6 ± 2.3%) did not differ between the groups (Figure 5C).

Discussion

Immune checkpoint proteins are extremely potent targets to modulate immunity in autoimmune diseases such as cardiovascular disease.[23,24,27] In this study, we show that stimulation of signaling through the immune checkpoint protein PD-1 inhibits atherosclerotic lesion development in WTD-fed LDLR$^{-/-}$ mice despite elevated serum cholesterol levels. This is accompanied by a decrease in inflammatory monocytes in peripheral blood in the early stages and a decrease in IFNγ-producing T cells, while atheroprotectiveIL-10 producing T cells, BREGS, B1 cells and OXLDL IgM levels were increased .

Previously, it was shown that absence of PD-1/PD-L1/2 signaling can aggravate atherosclerosis by enhancing T cell proliferation, activation of both CD4$^+$ as CD8$^+$ T cells, and more specifically by increasing pro-atherogenic IFNγ production by T cells.[21,22] In line with these findings, we show that agonistic PD-1 treatment resulted in a strongly impaired proliferative capacity of T cells, with a concomitant shift from TH1-associated IFNγ-producing CD4$^+$ cells towards anti-inflammatory IL-10 producing CD4$^+$ T cells. This was accompanied by a significant decrease in CD4$^+$ T cell numbers in the plaque. This is not surprising, as PD-1 signaling is known to suppress T cell activation and proliferation.[28,29] Reduced IFNγ-producing T cells in our agonistic PD-1 treated mice likely contributed to diminished atherosclerosis, as administration of exogenous IFNγ to APOE$^{-/-}$ mice resulted in an increase plaque formation,[30] while LDLR$^{-/-}$IFNγ$^{-/-}$ mice developed smaller plaques compared to control.[31] In contrast, IL-10 has well-described anti-atherogenic properties[32] and is often associated with regulatory T cells, which can suppress activation and proliferation of immune cells during atherosclerosis, including IFNγ-producing CD4$^+$ T cells.[33] Despite our observed reduction in T cell proliferation and IFNγ-producing T cells in agonistic PD-1 treated mice, we did not observe a difference in the percentage of FOXP3$^+$ CD4$^+$ T cells in the circulation (Figure S5A). That PD-1 directly acts on pro-atherogenic T cells without affecting Treg levels, was also described by Bu et al. who showed that PD-1 deficiency in LDLR$^{-/-}$ mice did not alter CD4$^+$FOXP3$^+$ T cells.[20]

Furthermore, we also observed a decrease in IFNγ-producing CD8$^+$ T cells, which are considered pro-atherogenic due to their cytotoxic capacity and inflammatory cytokine production.[34] This is in line with previous studies in which PD-1$^{-/-}$LDLR$^{-/-}$ mice showed an increase in pro-inflammatory cytokine expression, including IFNγ by CD8$^+$ T cells, and in which

PD-1 blockade induced IFNγ production by both $CD4^+$ and $CD8^+$ T cells.[21] Moreover, PD-1 expressing $CD8^+$ T cells from patients with atherosclerosis produced more anti-atherogenic IL-10 and less pro-atherogenic cytokines (IFNγ, TNFα) compared to $PD\text{-}1^-CD8^+$ T cells, further supporting a protective role for PD-1 in T cell-mediated immunity. Interestingly, $CD8^+$ T cells can also control monopoiesis and circulating monocyte levels in atherosclerosis,[35] which may have contributed to our observed reduction in circulating monocytes. The latter can also result from reduced T cell activation in PD-1 agonist treated mice as it has been shown that activated T cells can induce pro-inflammatory cytokine secretion by monocytes.[36] Lack of this monocyte activation upon PD-1 stimulation possibly prevents influx of new monocytes into the circulation. We show this reduction in monocytes is directly associated with PD-1 stimulation, as treatment with an isotype control resulted in comparable monocyte levels to PBS treatment. Due to limited statistical power in that particular experiment, no concrete conclusion can be drawn on atherosclerosis development. Agonistic PD-1 treatment was only able to delay WTD-induced monocytosis, as relative monocyte levels did not differ between control and PD-1 agonist treated mice after 6 weeks of treatment. Although we have no supportive evidence, the comparable levels of monocytes at sacrifice could be attributed to enhanced monocyte release from the bone marrow in PD-1 agonist treated mice to compensate for the reduced monocytes in the first few weeks of the treatment. As we also observe decreased monocyte levels in the spleen after 3 weeks of treatment, it is also a possibility that monocytes from the splenic reservoir[37] are suppressed during the initial weeks of PD-1 agonist treatment, resulting in reduced circulating monocytes. Notably, we did not observe a difference in macrophage content in the plaque, rendering it unlikely that the observed reduction in monocyte levels during the first weeks of the treatment is solely responsible for the observed plaque size reduction upon PD-1 agonism. However, a more detailed analysis regarding macrophage subsets in the plaque may shed more light on the underlying mechanisms involved.

The PD-1/PD-L1/L2 pathway is not only an important negative regulator of T cell responses but can also impact B cell immunity.[9,38] Although PD-L1 expressing B cells have been more extensively investigated, PD-1 is also upregulated on activated B cells and these $PD\text{-}1^+$ B cells have been linked to CD4 and CD8 T cell suppression previously.[9,38,39] Within 2 weeks upon PD-1 stimulation, we already observed elevated circulating $CD5^+CD1d^{hi}$ regulatory B cells, which could possibly exert an atheroprotective role by suppressing T cells. Previously, decreased amount of circulating BREGS were shown in patients with coronary atherosclerosis compared to healthy controls[40] and we showed that adoptive transfer of $IL\text{-}10^+$ BREGS in $LDLR^{-/-}$ mice reduced total leukocyte counts, lymphocytes, monocytes and activated T cells in circulation.[41] Notably, this adoptive transfer of $IL\text{-}10^+$ BREGS as well as lack of IL-10 producing B cells in $LDLR^{-/-}$ mice[42] did not affect plaque size, suggesting our observed increase in BREGS upon PD-1 agonism mainly contributes to reduced inflammation. Finally, we also found elevated levels of B1 cells in the circulation after PD-1 stimulation. B1 cells are considered atheroprotective via their production of primarily IgM natural antibodies directed at amongst others to OXLDL, which can prevent foam cell formation, and facilitate the clearance of apoptotic cells.[43] Corresponding to the increase in B1 cells, we observed elevated serum IgM levels and more specifically an absolute and relative increase in OXLDL-specific IgM, which has been shown to inversely relate to the incidence of CVD.[44]

Finally, we would like to address a few findings that warrant further research. First of all, our study was performed in female mice. Previous studies investigating the PD-1/PD-L1 pathway were performed in either male[22] or female mice, [21] and in both sexes PD-1 or PD-L1 deficiency aggravated atherosclerosis development. Therefore we do not anticipate sex differences upon PD-1 stimulation in our atherosclerosis model, although further research would be necessary to confirm this. Moreover, we also observed an enlargement of the spleen after PD-1 stimulation, while the amount of splenocytes, white pulp content and fat deposits is similar to those in control mice after 6 weeks of treatment. Previous studies using radioactively labeled monoclonal antibodies show that the spleen is a preferential site of accumulation for therapeutic antibodies,[45] which could possibly explain this observation. Finally, despite decreased atherosclerosis, we did observe elevated cholesterol levels in our PD-1 agonist treated mice. This is in contrast to Cochain et al. who found increased cholesterol levels upon PD-1 deficiency in $LDLR^{-/-}$ mice[22], while cholesterol levels remained unchanged in other studies investigating PD-1/PD-L1 deficiency in atherosclerosis.[20,21] It thus remains to be elucidated whether the observed effect on serum cholesterol levels in our study is directly related to PD-1 agonism or whether this is a secondary effect. Despite the increase

in cholesterol levels, we did observe a decrease in atherosclerosis development, suggesting that the atheroprotective effects that we observe on the immune system upon PD-1 treatment are effective in counteracting the increase in serum cholesterol levels. Further research investigating the direct effects on PD-1 agonism on cholesterol metabolism may provide mechanistic insights in this regard.

Given the recent advances in immune modulation as treatment for atherosclerosis,[46] our data show that PD-1 stimulation is a possible treatment to reduce atherosclerosis development. Although our study provides novel insights in the role of the PD-1 pathway in atherosclerosis, caution is warranted when using this target for treatment. While dampening the immune system and thus stimulation of the PD-1/PD-L1 axis is a potential pathway to reduce progression of atherosclerosis, activation of the immune system by blockade of the PD-1/PD-L1 pathway has shown promising results in the treatment of several cancers.[47] Indeed, a number of patients treated with anti-PD-1 or PD-L1 show cardiotoxicity, mostly myocarditis, especially in combination with anti-CTLA4 treatment.[48] Although more research is needed to enhance our knowledge regarding this ambivalence, recently it was reported that melanoma patients receiving anti-PD-1 and anti-CTLA4 treatment did not show a direct effect on atherosclerosis after 10 weeks of treatment.[49]

In conclusion, our data show that stimulation of the co-inhibitory PD-1 pathway inhibits atherosclerosis development via modulation of the immune response and supports that stimulation of co-inhibitory molecules can be a potential therapeutic strategy to limit atherosclerosis.

Figure 1 Short term PD-1 stimulation alters monocyte levels and inhibits T cell proliferation. (A) Experimental setup. LDLR$^{-/-}$ mice were fed a WTD or chow diet for 2 weeks while receiving an agonistic PD-1 antibody or control vehicle. (B) The weight and serum cholesterol levels were assessed before and after treatment. (C) Peripheral blood and (D) splenic monocyte percentages were measured by flow cytometry. (E) Proliferation of splenocytes after 3 days of stimulation with anti-CD3 and anti-CD28 measured by [3H]thymidine labeling (n=5/group). (F). Regulatory B cells in peripheral bloodwere measured by flow cytometry. Data are displayed as mean ± SEM. Statistics was performed using one-way ANOVA, with post-hoc comparison using Dunnett's multiple comparisons test, comparing control chow and PD-1 WTD groups to the control WTD group. P values ≤0.05 are considered significant. * P≤0.05, ** P≤0.01, *** P≤0.001.

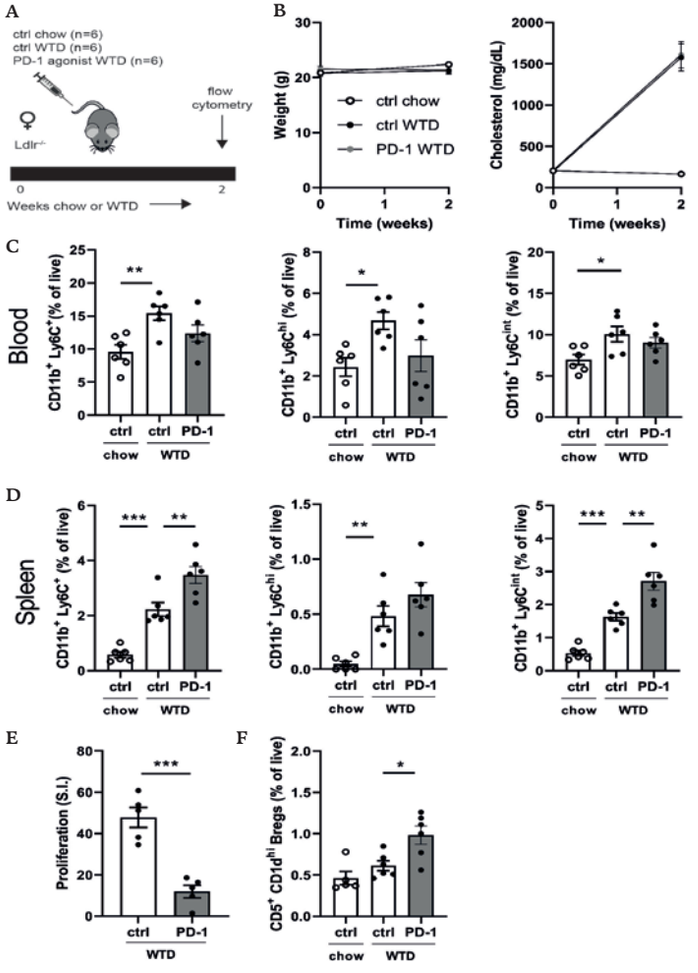


Figure 2 Agonistic PD-1 treatment inhibits WTD-induced increase in circulating monocytes. (A) Experimental setup. LDLR$^{-/-}$ mice were fed a WTD for 6 weeks while receiving an agonistic PD-1 antibody (n=15) or control vehicle (n=14). (B) Weight and serum cholesterol levels were assessed before, during and after treatment. Total monocytes (CD11b^{+}LY6G^{-}LY6C^{+}), inflammatory monocytes (CD11b^{+}LY6G^{-}LY6C^{high}) and patrolling monocytes (CD11b^{+}LY6G^{-}LY6C^{int}) were measured by flow cytometry in peripheral blood (C) and spleen (D). Mean ± SEM are shown. Statistics was performed using unpaired T-test. P values ≤0.05 are considered significant. * P≤0.05, *** P≤0.001, **** P≤ 0.0001.

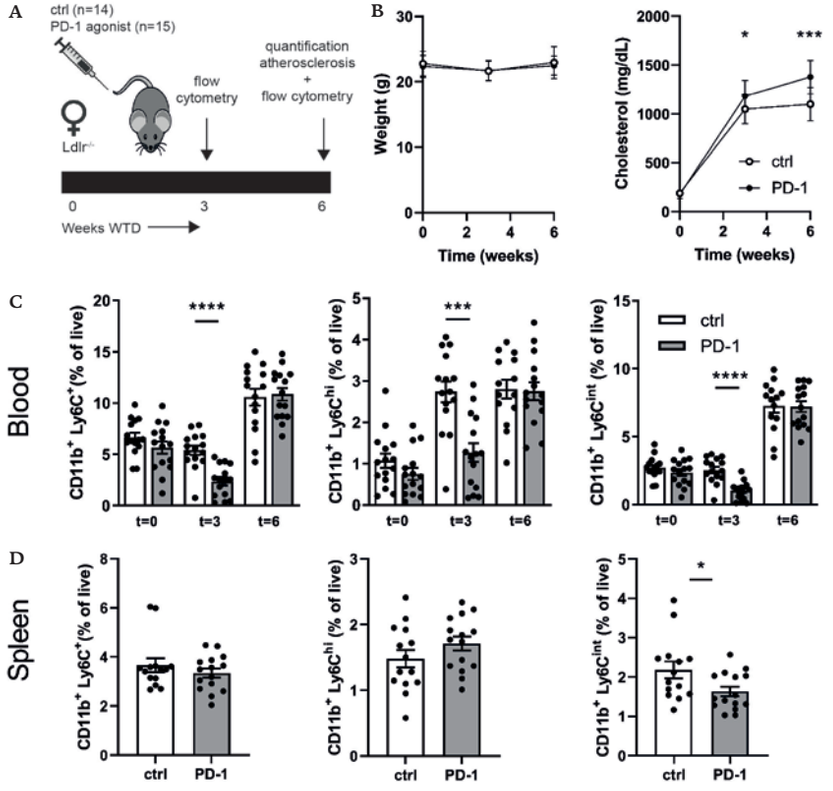


Figure 3 PD-1 stimulation promotes an anti-atherogenic T cell phenotype. (A) The amount of CD4^{+} and CD8^{+} T cells and their expression of CD69 was measured in splenocytes by flow cytometry. To measure intracellular cytokines, splenocytes were stimulated for 4 hours with PMA/ionomycin and BREFA. (B) Representative dotplots and (C) data graphs of intracellular IFNγ and IL-10 within CD4^{+} and CD8^{+} T cells are shown. (D) CD4^{+} T cell numbers in the atherosclerotic plaque were manually scored. Scale bars indicate 50µm. Mean ± SEM are shown. Statistics was performed using unpaired T-test. P values ≤0.05 are considered significant. ** P≤0.01.

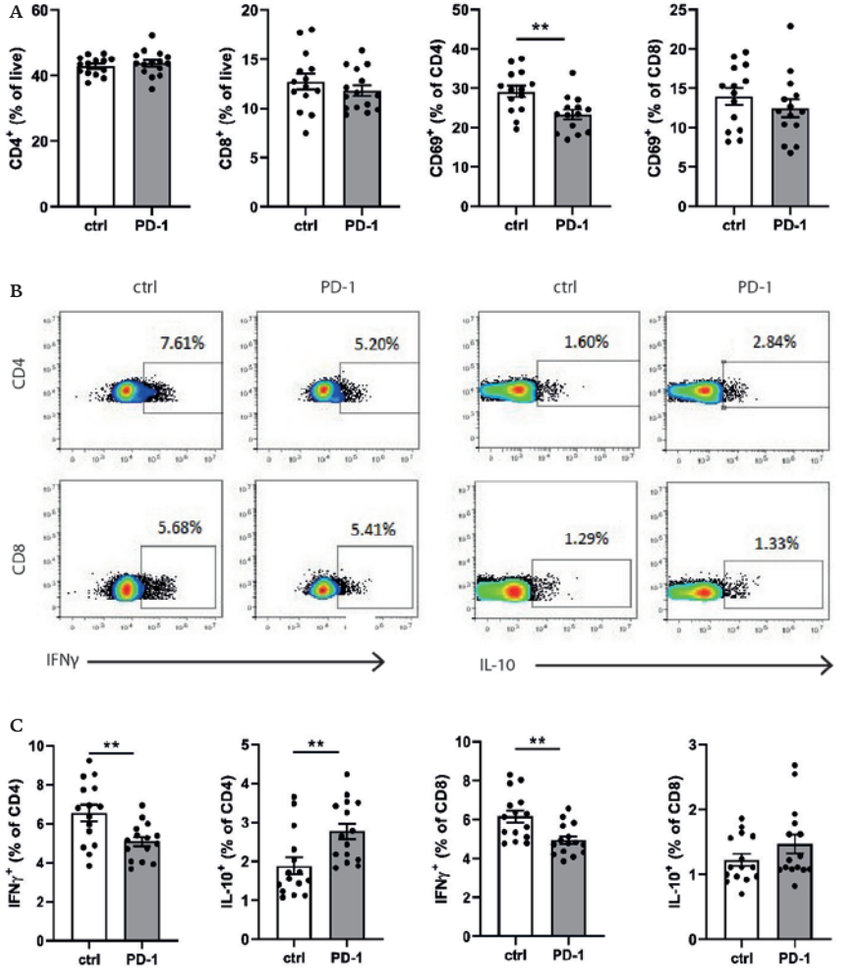


Figure 4 Elevated B1 cells and OXLDL-IgM levels in agonistic PD-1 treated mice. Total CD19$^+$ B cells, and B1 cells (CD19$^+$B220^{lo}) and regulatory B cells (CD19$^+$CD5$^+$CD1d^{hi}) were measured by flow cytometry in blood (A). Total serum IgM (µg/ml), CU-OXLDL IgM (relative light units (RLU)/100ms) and the CU-OXLDL IgM/total IgM ratio measured by ELISA (B). Mean ± SEM are shown. Statistics was performed using unpaired T-test. P values ≤0.05 are considered significant. * P≤0.05, ** P≤0.01, *** P≤0.001, **** P≤ 0.0001

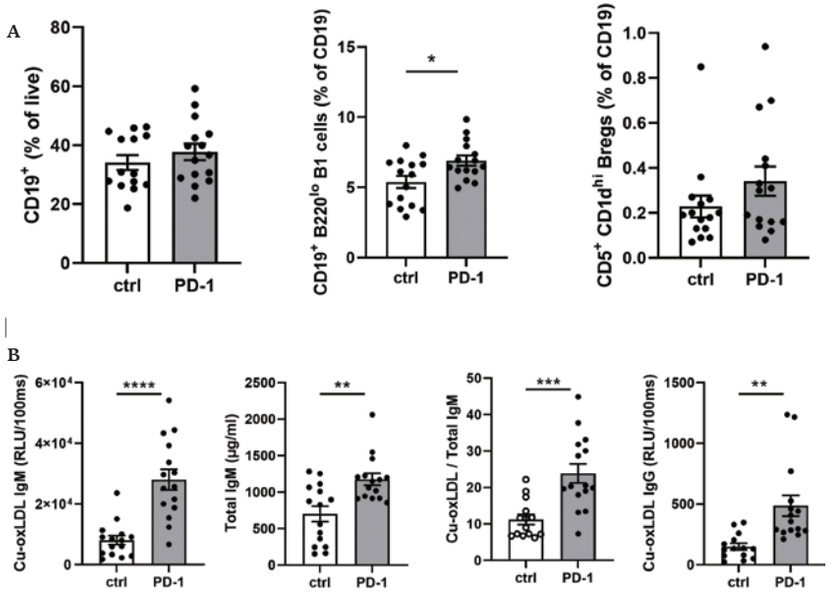
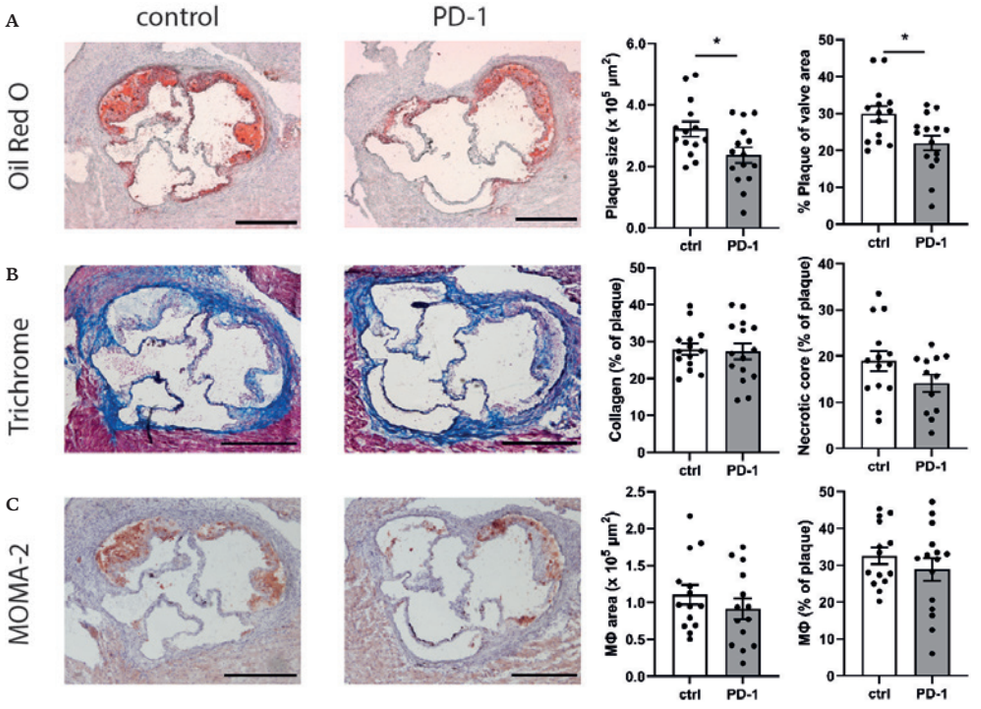


Figure 5 PD-1 stimulation inhibits atherosclerosis development. Aortic root plaque size and composition was determined using an Oil-Red-O staining and representative images of the stainings are shown. Data is shown as total plaque size, and as percentage of total valve area (A). Collagen content and necrotic core size were determined using trichrome staining, and are showed as percentage of the total plaque (B). Macrophage content was determined using MOMA-2 antibody staining, and is shown as total MOMA-2 positive area, as well as the percentage of total plaque (C). Scale bars indicate 250µm. Mean ± SEM are shown. Statistics was performed using unpaired T-test. P values ≤0.05 are considered significant. * P≤0.05.



All mentioned supplementary figures and tables in this chapter can be found on the corresponding website by scanning this QR code.

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CHAPTER 10

General discussion

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 $CD4^{+}CD28^{null}$ 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 $CD28^{null}$ 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

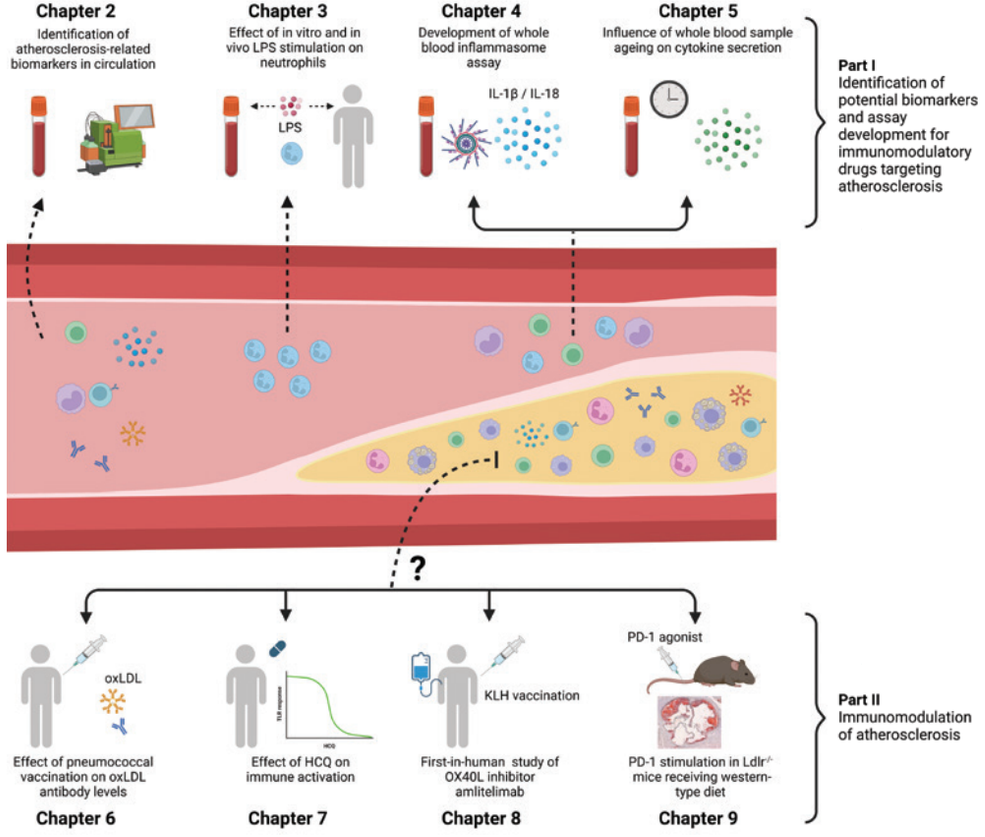
the onset of atherosclerosis, but they are also implied in plaque destabilization.[17-19] Currently, no treatments specifically targeting neutrophils have been registered, although this could be a valuable method to prevent cardiovascular events by stabilizing atherosclerotic plaques. To be able to test 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 performedthe 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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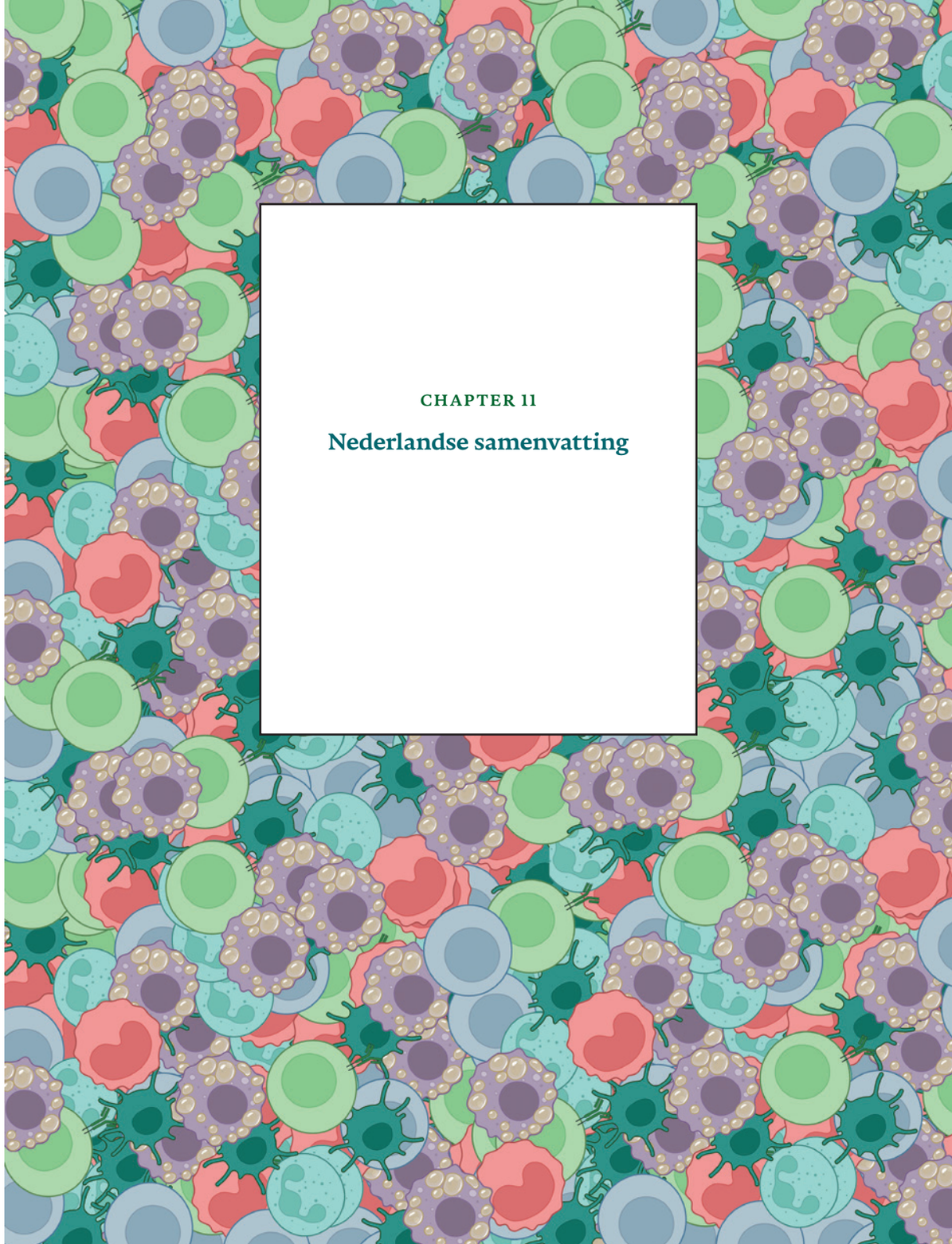

CHAPTER 11

Nederlandse samenvatting

Hart- en vaatziekten zijn wereldwijd de belangrijkste doodsoorzaak.[1] Hart- en vaatziekten omvatten alle aandoeningen van het hart en het vaatsysteem, waaronder onder andere beroertes, hartritmestoornissen, hartfalen, aangeboren hartafwijkingen en hartinfarcten vallen. De belangrijkste onderliggende oorzaak voor hart- en vaatziekten is atherosclerose, ook wel slagaderverkalking genoemd. Atherosclerose wordt gekenmerkt door de vorming van een plaque, voornamelijk bestaande uit lipiden en ontstekingscellen, in de vaatwand. Uiteindelijk kan deze plaquevorming leiden tot atherotrombose of stenose van het bloedvat wat resulteert in een hartinfarct, longembolie of beroerte en uiteindelijk tot de dood kan leiden.

De ontwikkeling van atherosclerose begint al bij jongvolwassenen,[2,3] en ontwikkelt zich langzaam in de meeste mensen. Er zijn diverse risicofactoren die de ontwikkeling van atherosclerose beïnvloeden. Sommige zijn aanpasbaar, zoals levensstijl, waaronder roken en dieet, terwijl andere niet beïnvloedbaar zijn, zoals leeftijd en erfelijke factoren. De ontwikkeling van atherosclerose begint met een disbalans in cholesterolwaarden in het bloed, voornamelijk een verhoging van de hoeveelheid lipoproteïnen met een lage dichtheid (low density lipoproteins, LDL). LDL accumuleert in de subendotheliale ruimte in de vaatwand op plaatsen waar een hoge verstoring in de bloedsomloop is, zoals in de cardiovasculaire bloedvaten. Momenteel bestaat de behandeling van atherosclerose, naast chirurgisch ingrijpen bij acute klinische klachten, uit het gebruik van cholesterolverlagende middelen. Daarnaast wordt levensstijlverandering geadviseerd en worden anticoagulantia en bloeddrukverlagende middelen voorgeschreven. Hoewel atherosclerose primair een ziekte is die wordt veroorzaakt door lipiden, speelt het immuunsysteem ook een belangrijke rol tijdens de ontwikkeling van atherosclerose. Daarom wordt atherosclerose tegenwoordig ook gezien als een chronische ontstekingsziekte. De behandeling met cholesterolverlagende middelen is maar gedeeltelijk succesvol, aangezien de kans op terugkerende cardiovasculaire events groot blijft.[4] Dit benadrukt de noodzaak voor additionele therapieën om atherosclerose te behandelen. Bij de ontwikkeling van nieuwe therapeutische strategieën is het essentieel om de rol van het immuunsysteem tijdens de ontwikkeling van atherosclerose, en het micromilieu van de plaque zelf, te ontrafelen om nieuwe diagnostische tools en behandelingen voor atherosclerose te kunnen ontwikkelen.

HET IMMUUNSYSTEEM IN ATHEROSCLEROSE

Zoals hierboven genoemd, begint atherosclerose met een ophoping van LDL in de vaatwand. In de vaatwand wordt LDL geoxideerd (OXLDL), en hierbij wordt het immuunsysteem geactiveerd. In eerste instantie spelen macrofagen een grote rol. Ze fagocyteren OXLDL en differentiëren hierdoor naar pro-inflammatoire schuimcellen.[5,6] Naast OXLDL kunnen ook cholesterolkristallen zich ophopen in macrofagen wat leidt tot activatie van het inflammasoom,[7,8] een eiwit dat resulteert in productie en uitscheiding van het cytokine interleukine(IL)-1β en verdere inflammatie. Naast macrofagen migreren neutrofielen, ook onderdeel van het aangeboren immuunsysteem, in een vroeg stadium naar de plaque. Na neutrofielactivatie vindt er opregulatie van adhesiemoleculen plaats wat dysfunctie van het endotheel verergert.[9] Tijdens de eerste stadia van de ontwikkeling van atherosclerose speelt het aangeboren immuunsysteem een belangrijke rol. In latere stadia wordt het verworven immuunsysteem ook geactiveerd. In de plaque zijn T-cellen te vinden, die bijvoorbeeld OXLDL-specifieke epitopen herkennen.[10,11] Daarnaast spelen B-cellen ook een rol bij atherosclerose, onder andere door de productie van antilichamen tegen OXLDL.

Niet alle immuuncellen spelen een atherogene rol tijdens de ontwikkeling van atherosclerose. Sommige immuuncellen remmen de ontwikkeling van atherosclerose, voornamelijk door het onderdrukken van de immuunrespons. Regulatoire T- en B-cellen bijvoorbeeld, remmen activatie van het verworven immuunsysteem, voornamelijk door de uitscheiding van het anti-inflammatoire cytokine IL-10. Daarnaast kunnen B-cellen atherosclerose remmen door de productie van antilichamen tegen OXLDL van het immunoglobuline(IG)M type. Wanneer OXLDL-IgM bindt aan OXLDL, wordt de opname van OXLDL door macrofagen geblokkeerd en worden de partikels uiteindelijk geklaard door de lever.[12-14] In tegenstelling tot OXLDL-IgM zorgt OXLDL-specifiek IgG er juist voor dat OXLDL sneller wordt opgenomen door macrofagen, en versterkt het daarmee de plaque ontwikkeling.[15]

IMMUNOMODULATIE VAN ATHEROSCLEROSE

Aangezien het immuunsysteem een belangrijke rol speelt in de ontwikkeling van atherosclerose, is modulatie van het immuunsysteem een potentieel belangrijke methode om cardiovasculaire events te voorkomen. Recent

zijn de eerste klinische studies uitgevoerd in patiënten met hart- en vaatziekten naar het effect van immunomodulatie op terugkerende cardiovasculaire events. De eerste klinische studie die effect liet zien is de CANTOS (canakinumab antiinflammatory thrombosis outcome study) trial. Tijdens deze studie zijn patiënten, die zijn opgenomen voor een hartinfarct en met een hsCRP level >2mg/L (een indicatie voor ontsteking), behandeld met canakinumab, een antilichaam dat IL-1β blokkeert, of een placebo.[16] De studie toonde aan dat de patiënten behandeld met canakinumab minder kans hadden op een terugkerend cardiovasculair event. Behandeling met colchicine, een breed immunosuppressief middel, resulteerde in een lagere kans op een terugkerend cardiovasculair event.[17] Helaas liet de CANTOS trial ook zien dat patiënten behandeld met canakinumab een hogere kans hadden op een fatale infectie, wat aangeeft dat de balans tussen immuunactivatie en immuunsuppressie precair is, en dat immunomodulatie voorzichtig moet worden toegepast en bij voorkeur specifiek voor atherosclerose.

DOEL VAN HET ONDERZOEK

Het doel van het onderzoek beschreven in dit proefschrift is het ontwikkelen van diagnostische handvaten en potentiële biomarkers gericht op de identificatie van immunomodulatoire medicijn-effecten in toekomstige klinische studies. Daarnaast is er onderzoek verricht naar nieuwe immunomodulatoire strategieën om atherosclerose te remmen, zowel in muizen als in de mens.

Deel I: Identificatie van potentiële atherosclerose gerelateerde biomarkers, en ontwikkeling van assays voor gebruik in klinische studies

In Hoofdstuk 2 is een observationele klinische studie beschreven waarin het effect van leeftijd en roken, beiden risicofactoren voor hart- en vaatziekten, op het immuunsysteem is onderzocht. In deze studie zijn vijf groepen vrijwilligers geïncludeerd. Dit zijn jonge, gezonde vrijwilligers (18-25 jaar, n=30), gezonde ouderen (>60 jaar, n=20), jonge rokers (18-25 jaar, n=20), zware rokers (>45 jaar, n=11) en patiënten met coronaire hartziekten (>60 jaar, n=27). In deze groepen werden circulerende immuuncellen geanalyseerd met flowcytometrie, monocyt- en T-cel responsen na volbloed stimulatie gemeten en proteomics gebruikt om inflammatoire eiwitten te meten. Ondanks kleine groepsgroottes, waren er duidelijke effecten te zien van zowel veroudering als roken op het immuunsysteem.

In ouderen was een lager niveau van totaal $CD8^+$ T-cellen en naïeve $CD4^+$ en $CD8^+$ T-cellen te zien, met een verhoging in $CD4^+$ en $CD8^+$ effector geheugencellen. Daarnaast werd een verhoogd niveau gevonden van de pro-inflammatoire en chemotactische eiwitten TREM1 en CCL11 in ouderen. Het immuunsysteem van zware rokers was in het algemeen in een verhoogd geactiveerde staat, met verhoogde neutrofiel-aantallen, een hoger percentage TH1-cellen en interferon (IFN)γ-producerende T-cellen, en minder regulatoire B-cellen. Het immuunsysteem van de hartpatiënten leek over het algemeen op dat van de gezonde ouderen, waarschijnlijk door het gebruik van immunomodulatoire medicatie, zoals statines. Wel was het atheroprotectieve OXLDL-specifieke IgM significant verlaagd in patiënten, in vergelijking met gezonde jongeren. Deze data laten zien dat in relatief kleine groepen duidelijke immunologische verschillen tussen populaties aan te tonen zijn, wat deze populaties (voornamelijk ouderen en zware rokers) potentieel geschikt maakt als 'modelpopulatie' in klinische studies, afhankelijk van het medicijn dat getest gaat worden. In hoofdstuk 3 wordt onderzoek naar het effect van lipopolysacchariden- (LPS) stimulatie op neutrofielen beschreven. Neutrofielen spelen een rol in het beginstadium van atherosclerose zoals hierboven beschreven, maar neutrofielen spelen ook een belangrijke rol bij de destabilisatie van de plaque.[18-20] Om nieuwe medicijnen gericht op neutrofielfunctie te testen in gezonde vrijwilligers, is activatie van neutrofielen nodig aangezien neutrofielen in deze populatie onder basale omstandigheden niet geactiveerd zijn. Hiervoor kan een intraveneuze toediening van LPS gebruikt worden, die zorgt voor een korte inflammatoire respons van enkele uren. In het onderzoek in hoofdstuk 3 wordt beschreven dat deze LPS-toediening zorgt voor activatie van neutrofielen, gemeten aan de hand van activatiemarkers met flowcytometrie en de uitscheiding van eiwitten uit de granules van neutrofielen in het plasma. Deze metingen werden ook uitgevoerd in LPS-gestimuleerde volbloedkweken van gezonde vrijwilligers. De effecten van de *in vitro* stimulatie komen grotendeels overeen met die van de *in vivo* stimulatie. Op basis van deze uitkomsten kunnen de *in vitro* en *in vivo* LPS-stimulaties toegepast worden in toekomstige klinische onderzoeken met nieuwe neutrofiel-gerichte middelen. In hoofdstukken 4 en 5 wordt de ontwikkeling van volbloedassays beschreven, wat waardevolle assays zijn om te gebruiken in klinische studies om medicatie-effecten te meten. In hoofdstuk 4 wordt de ontwikkeling van een volbloed NLRP3 inflammasoom assay beschreven. Zoals eerder al genoemd,

speelt het inflammasoom een belangrijke rol tijdens de ontwikkeling van atherosclerose, en inhibitie van IL-1β (geproduceerd na inflammasoom activatie) was succesvol in het verlagen van de kans op een terugkerend cardiovasculair event. Voor toekomstige klinische studies gericht op (modulatie van) inflammasoom activiteit is een goede assay van belang. In de assay beschreven in hoofdstuk 4 wordt volbloed eerst gestimuleerd met LPS, om cellen aan te zetten tot inflammasoom-activatie, gevolgd door toevoeging van ATP als tweede stimulus om het inflammasoom volledig te activeren. We laten zien dat deze assay geschikt is om inflammasoom-activatie, en de daaropvolgende IL-1β- en IL-18-productie, te meten terwijl het bloed minimaal verdund wordt. Hierdoor is het een geschikte assay voor gebruik tijdens klinische studies om medicijneffecten te monitoren. In hoofdstuk 5 werd de volbloedassay verder gekarakteriseerd. In dit hoofdstuk is gekeken naar het effect van veroudering van bloedmonsters op de cytokineproductie door monocyten en lymfocyten. Volbloed werd gestimuleerd met LPS om monocyten te activeren, of PMA + ionomycine of SEB om lymfocyten te activeren. Dit werd gedaan direct na bloedafname tot maximaal 24 uur na bloedafname. De respons van monocyten verminderde snel gedurende de veroudering, na 30 minuten is de uitscheiding van cytokinen al verlaagd met 20–50%, verder dalend tot 80–100% 10 uur na bloedafname. De respons van lymfocyten was veel stabieler, de uitscheiding van cytokinen was verlaagd met 20–50% 24 uur na bloedafname. Het toevoegen van kweekmedium, en daarmee nutriënten, herstelde het vermogen tot cytokineproductie niet, wat laat zien dat de geobserveerde vermindering waarschijnlijk niet door een tekort aan nutriënten kwam. Daarnaast bleef de hoeveelheid cellen die cytokinen produceren gelijk, wat duidt op een afname van cytokineproductie en niet het afsterven van cellen. Deze data laten zien dat snelle monsterverwerking belangrijk is wanneer monocytresponsen worden gemeten in volbloedkweken.

DEEL II: IMMUNOMODULATIE VAN ATHEROSCLEROSE

In Hoofdstuk 6 wordt een klinische studie beschreven waarin gezonde vrijwilligers werden gevaccineerd met het pneumokokkenvaccin Prevenar-13 of een placebo om de effecten van vaccinatie op circulerende antilichamen tegen OXLDL en phosphorylcholine (PC) te meten. In muizen is beschreven dat immunisatie van muizen met *Streptococcus pneumoniae* resulteerde in een verhoging van het circulerende OXLDL-specifieke IgM-gehalte en daardoor een verlaging van atherosclerose-ontwikkeling.[21] Deze toename in OXLDL-specifiek IgM komt door de aanwezigheid van PC in de celwand van *Streptococcus pneumoniae* dat moleculaire overeenkomsten vertoont met OXLDL. Gezonde vrijwilligers werden gerandomiseerd om 0, 1, 2 of 3 vaccinaties te krijgen met Prevenar-13, en OXLDL, PC-antilichamen en lipidengehaltes in het serum werden gemeten vanaf baseline tot 68 weken na de eerste dosis. Helaas werd er geen effect gevonden van vaccinatie op de eerdergenoemde eindpunten. Dit kan liggen aan het feit dat het Prevenar-13-vaccin bestaat uit specifieke peptiden van *Streptococcus pneumoniae* in tegenstelling tot de immunisatie in muizen, waarbij de volledige bacterie gebruikt werd. Hierdoor is mogelijk de hoeveelheid PC, en dus de antilichaamrespons, tegen het vaccin lager. Daarnaast kan het zijn dat de situatie in de muis niet vertaalbaar is naar de situatie in de mens. In Hoofdstuk 7 zijn de effecten van hydroxychloroquine (HCQ) op zowel *in vitro* als *in vivo* celactivatie bestudeerd. HCQ is een breed immunosuppressief medicijn, dat gebruikt wordt voor de behandeling van malaria, en diverse auto-immuunziekten. Een klinische studie naar het effect van HCQ op terugkerende cardiovasculaire events is momenteel gaande.[22] Het exacte werkingsmechanisme van HCQ en de dosisafhankelijkheid van de immunosuppressieve effecten is nog onbekend. In deze studie hebben werden immuunsuppressie-assays uitgevoerd met immuuncellen van gezonde vrijwilligers die oraal HCQ toegediend kregen, en vergeleken met celkweken met perifeer bloed mononucleaire cellen (PBMCs), waarbij HCQ direct aan de cellen toegevoegd werd. Het effect van HCQ op endosomale TLR-stimulatie werd onderzocht door PBMCs te stimuleren met TLR3-, -7-, en -9- liganden en vervolgens IFNα- en IL-6-productie te meten. Daarnaast zijn de effecten op T- en B-celproliferatie gemeten. In de *in vitro* experimenten werden duidelijke suppressie van zowel TLR-responsen als B-cel-proliferatie gevonden bij concentraties >1000ng/mL. Er werd geen effect op T-cel-activatie en proliferatie gevonden, wat mogelijk veroorzaakt werd door de gekozen stimulus. Deze was mogelijk te sterk om een medicatie-effect te kunnen meten. De concentraties HCQ die gevonden werden in plasma van HCQ-gedoseerde proefpersonen, waren lager dan de concentraties waarbij effect te zien was *in vitro*. Hierdoor was er ook weinig immuunsuppressie in de *ex vivo* cellulaire assays. Ondanks dat er geen immuun suppressie te zien was in onze gedoseerde proefpersonen, werkt HCQ wel bij de behandeling van auto-immuunziekten. Dit komt waarschijnlijk doordat de lokale concentraties HCQ

in de weefsels en intracellulair hoger zijn dan wat we in plasma kunnen meten. Hoofdstuk 8 beschrijft een klinische trial waarbij het nieuwe medicijn amlitelimab (KY1005), een antilichaam dat OX40L blokkeert, is getest in gezonde vrijwilligers. OX40L wordt tot expressie gebracht op antigeen-presenterende cellen en speelt een rol bij de activatie van T-cellen: het is een zogenaamde co-stimulatie-factor die nodig is voor volledige activatie van T-cellen via binding aan OX40. Om te testen of amlitelimab zowel klinisch veilig is als ook daadwerkelijk immuunsuppressief werkt, werd vaccinatie met het neo-antigen keyhole limpet hemocyanin (KLH) toegepast. Ook is het effect op een herhaalvaccinatie met tetanus getest. Eerder is in muizen aangetoond dat het blokkeren van de OX40-OX40L signalering atherosclerose significant remt.[23,24] Blokkeren van OX40L in de mens zou dus ook mogelijk een goede methode zijn om atherosclerose te remmen. De toediening van amlitelimab leidde alleen tot milde bijwerkingen. Amlitelimab-behandeling verlaagde de KLH-specifieke IgG-productie, maar niet significant. Daarnaast was de respons op een intradermale KLH-injectie na vaccinatie significant verlaagd in de vrijwilligers die amlitelimab kregen ten opzichte van placebo. Deze resultaten laten zien dat amlitelimab veilig is om toe te dienen, en ook in de mens de immuunrespons remt. Hoofdstuk 9 beschrijft een muizenstudie waarbij het effect van stimulatie van het immuuncheckpoint-eiwit PD-1 op atherosclerose ontwikkeling is getest. PD-1 komt tot expressie op geactiveerde T- en B-cellen en bij binding aan hun ligand (PD-L1 of PD-L2) wordt de T- of B-cel geremd, een zogenaamd co-inhibitoir signaal. Eerdere studies in muizen hebben aangetoond dat inhibitie van het PD-1 signaal resulteerde in verergering van atherosclerose-ontwikkeling, wat leidde tot de hypothese dat stimulatie van het PD-1-signaal ontwikkeling van atherosclerose zal remmen.[25-27] Voor dit experiment zijn $LDLR^{-/-}$ muizen gebruikt. Deze muizen missen de receptor voor LDL, waardoor de hoeveelheid cholesterol in het bloed stijgt wanneer deze muizen een dieet met een hoog cholesterolgehalte krijgen. Hierdoor ontwikkelen deze muizen atherosclerose. Stimulatie van PD-1 met een agonistisch antilichaam resulteerde inderdaad in een verlaging van atherosclerose-ontwikkeling. Daarnaast zagen we dat de monocytinflux in het bloed, veroorzaakt door hoge cholesterolgehaltes, werd vertraagd. Ook was er een verhoging van B1-cellen in het bloed met daarbij behorende verhoging van OXLDL-specifiek IgM en een verlaging van pro-inflammatoir IFNγ producerende T-cellen en verhoging van T-cellen die het anti-inflammatoire cytokine IL-10 produceren. Deze resultaten tonen aan dat stimulatie van het PD-1-signaal inflammatie remt en daardoor de atherosclerose-ontwikkeling.

PERSPECTIEVEN

Momenteel lopen patiënten met hart- en vaatziekten veroorzaakt door atherosclerose een groot risico op (terugkerende) cardiovasculaire events. Om dit risico te verkleinen zijn nieuwe geneesmiddelen voor atherosclerose nodig. Aangezien het immuunsysteem een belangrijke rol speelt tijdens de ontwikkeling van atherosclerose, lijkt immunomodulatie een belangrijke stap richting deze nieuwe behandelmethoden. Er zijn enkele klinische studies uitgevoerd waarvan er twee succesvol waren, namelijk het gebruik van canakinumab (een IL-1β inhibitor) en colchicine.[16,17,28] Beide medicijnen hebben een brede immunosuppressieve werking. Het risico daarbij is dat er bijwerkingen zijn. Dit is bijvoorbeeld geïllustreerd in de studie met canakinumab, waarbij het risico op (fatale) infecties groter was in vergelijking met placebo.[16] Om de kans op bijwerkingen zo klein mogelijk te houden is het nodig om medicijnen te hebben die specifiek atherosclerose remmen. Daarnaast zijn niet alle patiënten met hart- en vaatziekten hetzelfde: het risico op een terugkerend cardiovasculair event is verschillend tussen patiënten. Zo waren patiënten met een verhoogde waarde van hs-CRP meer gebaat bij behandeling met canakinumab dan patiënten met lagere ontstekingswaarden.[29]

Op het moment richten klinische studies zich vooral op het voorkomen van terugkerende cardiovasculaire events, maar idealiter zou een primair cardiovasculair event voorkomen de voorkeur hebben. Hiervoor is een behandeling nodig, die specifiek is voor de individuele patiënt en zonder (serieuze) bijwerkingen. Het is lastig te voorspellen welke personen risico lopen op een cardiovasculair event aangezien vrijwel iedereen in meer of mindere mate atherosclerose ontwikkelt gedurende zijn/haar leven. Het verhogen van OXLDL-specifiek IgM lijkt een ideale methode. In diverse muizenstudies is namelijk gebleken dat dit de ontwikkeling van atherosclerose remt,[21] en ook in de mens is aangetoond dat het niveau van OXLDL-IgM omgekeerd correleert met de hoeveelheid atherosclerose.[30] Vaccinatie zou alleen OXLDL-specifiek IgM moeten verhogen, en niet IgG, aangezien OXLDL-specifiek IgG juist atherosclerose-ontwikkeling stimuleert via de verhoogde opname van OXLDL door macrofagen. Ook in de mens is OXLDL-specifiek IgG gecorreleerd met atherosclerose.[31,32] Een

behandeling of vaccinatie zou zich dus vooral moeten richten op de B1-cellen, die verantwoordelijk zijn voor productie van autoantilichamen van het IgM-subtype. Een andere methode kan zijn om class-switching naar het IgG-subtype te blokkeren tijdens vaccinatie. Dit zou mogelijk gedaan kunnen worden met het blokkeren van de OX40-OX40L signalering, OX40 speelt namelijk een belangrijke rol bij class-switching van IgM naar IgG. [33]

CONCLUSIES

Het voorkomen van de progressie van atherosclerose en destabilisatie van de plaque is nodig om (terugkerende) cardiovasculaire events te voorkomen. Hiervoor is modulatie van het immuunsysteem een veelbelovende methode. Het onderzoek beschreven in dit proefschrift kan bijdragen aan de ontwikkeling van een dergelijke therapie. In deel I is de ontwikkeling van volbloedassays en potentiële biomarkers beschreven die bij kunnen dragen aan toekomstig onderzoek naar immunomodulatoire medicijnen in klinische studies. In deel II zijn nieuwe strategieën voor immunomodulatie in atherosclerose getest. Op het moment zijn er geen immunomodulatoire therapieën beschikbaar voor de behandeling van atherosclerose, maar deze komen waarschijnlijk beschikbaar in de niet al te verre toekomst.

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Curriculum Vitae

Wieke Grievink werd geboren op 31 mei 1986 in Lichtenvoorde. In juni 2003 behaalde zij haar HAVO-diploma aan het Schaersvoorde in Aalten. In datzelfde jaar begon zij aan de studie Biologie & Medisch Laboratoriumonderzoek aan het Saxion college in Enschede. Tijdens deze studie deed ze twee stages, de eerste aan het Universitair Medisch Centrum Groningen, de tweede aan de Wageningen Universiteit bij de afdeling Microbiologie onder begeleiding van dr. S. Fuentes en prof. dr. H. Smidt. Deze stage werd afgerond met het verslag '*Isolation and characterization of an unknown Clostridium species from the gastro-intestinal tract of a rat*'. Na het behalen van haar Bachelortitel in 2007 heeft ze op diverse laboratoria als analist gewerkt: in 2008 bij Sanquin op het Onderzoek & Ontwikkelingslab, van november 2008 tot februari 2013 bij Crossbeta Biosciences in Utrecht (productie van stabiele Aβ1-42 oligomeren), en vanaf 2013 tot april 2016 bij Good Biomarker Sciences te Leiden, waar ze laboratoriumanalyses uitvoerde voor klinische onderzoeken. Vanaf juni 2016 is ze werkzaam bij Centre for Human Drug Research (CHDR) als Bioanalytical Scientist. In oktober 2018 is ze begonnen als promovenda bij het CHDR onder begeleiding van dr. M. Moerland en prof. dr. J. Burggraaf, en de afdeling BioTherapeutics van het Leiden Academic Centre for Drug Research onder begeleiding van dr. A.C. Foks, dr. I. Bot en prof. dr. J. Kuiper.