Profiling of proteins and targeting of myeloid mechanisms in atherosclerosis

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Chapter 1

General introduction

General introduction

Cardiovascular disease

Cardiovascular diseases (CVDs) predominate mortality worldwide[1]. CVDs are diseases affecting the heart and arteries, such as coronary heart disease, cerebrovascular disease and peripheral arterial disease[1]. CVDs are generally caused by atherosclerosis, a lipid-driven inflammatory disease characterized by accumulation of lipids and immune cells forming plaques in large- and middle-sized arteries[2]. These plaques can reduce the blood flow and can develop into advanced rupture-prone plaques. Upon physical rupture of these plaques, a thrombus can be formed, which can completely block the artery and subsequently cause a stroke or myocardial infarction[3,4]. Myocardial infarction and stroke account for 85% of CVD deaths[1].

Treatment limiting progression of atherosclerosis is currently aimed at decreasing plasma cholesterol levels with for example statins and proprotein convertase subtilisin-kexin type 9 (PCSK9) inhibitors, which can significantly lower cardiovascular events[5–7]. However, patients can have a residual inflammatory risk, generally indicated by high-sensitivity C-reactive protein (hsCRP) levels of ≥2 mg per liter, which significantly associates with major adverse cardiovascular events and cardiovascular mortality[8–10]. In 2017, the Canakinumab Antiinflammatory Thrombosis Outcome Study (CANTOS) trial illustrated the potential of an anti-inflammatory therapy for atherosclerosis. In this trial a therapeutic monoclonal antibody canakinumab, which targets interleukin-1β (IL-1β), was administered to patients with ≥2 mg hsCRP/liter and prior myocardial infarction. Canakinumab reduced the incidence of secondary cardiovascular events significantly without affecting lipid levels, demonstrating the effect of anti-inflammatory therapy. However, canakinumab did not affect all-cause mortality and led to significantly more deaths due to infection, necessitating investigation of other anti-inflammatory therapies[11].

Besides the CANTOS trial, various other anti-inflammatory trials have been conducted in the past years. In the Virginia Commonwealth University Anakinra Remodeling Trial 3 (VCUART3), patients with ST-segment-elevation myocardial infarction were treated with anakinra, a recombinant interleukin-1 receptor antagonist, for two weeks. HsCRP was significantly lowered in the anakinra group versus placebo in the first 14 days. Also, the incidence of new-onset heart failure or death or the incidence of death and hospitalization because of heart failure was significantly reduced with anakinra compared to placebo. However, the composite of recurrent ischemic events, which included death, recurrent myocardial infarction or urgent revascularization, did not differ between the anakinra and placebo group[12]. The anti-inflammatory agent colchicine has been tested as a cardiovascular therapy as well. Colchicine exerts its effects mainly via the disruption of tubulin, which results in various anti-inflammatory effects[13]. In the two low-dose colchicine (LoDoCo) trials, the occurrence of cardiovascular events in coronary disease patients was significantly reduced by a dose of 0.5 mg colchicine per day[14,15]. This colchicine dose also reduced the risk of cardiovascular events in patients that suffered from a recent myocardial infarction, in the Colchicine Cardiovascular Outcomes Trial (COLCOT)[16]. Nowadays, this low dose of colchicine (0.5 mg per day) is approved by the U.S. Food and Drug Administration for atherosclerotic disease patients[17,18]. However, in the CLEAR trial, the risk of cardiovascular

events was not affected by colchicine in patients with a previous myocardial infarction[19]. Furthermore, the COLCOT trial revealed a higher incidence of pneumonia in patients receiving colchicine compared to placebo[16]. Together, these side-effects render additional research and developments in anti-inflammatory therapies for CVDs necessary. Therefore, up- as well as downstream molecules of IL-1β and colchicine targets are currently a focus for atherosclerosis therapy development. IL-6 is a target downstream of IL-1β, and IL-6 inhibition by the antibody ziltivekimab in the two Phase 2 trials RESCUE and RESCUE-2 in patients at high atherosclerotic risk reduced hsCRP levels from baseline ≥2mg/L hsCRP. Serum amyloid A and fibrinogen, which are inflammatory and thrombotic biomarkers respectively, were reduced as well[20,21]. Currently, in the ZEUS trial, the Ziltivekimab Cardiovascular Outcomes Study, patients with cardiovascular disease, systemic inflammation and chronic kidney disease are treated with ziltivekimab to determine the effect on the occurrence of cardiovascular events[22,23]. Furthermore, the IL-1 receptor accessory protein (IL1RAP) is the co-receptor for, among others, IL-1β. The humanized antibody against this co-receptor, CAN10 (Cantargia AB), inhibits IL-1, IL-33 and IL-36 activity and is therefore of interest as therapeutic against atherosclerosis[24] and currently in a Phase 1 clinical trial for safety[25]. Another potential upstream target of IL-1β is the NLRP3 (NOD [nucleotide oligomerization domain]-, LRR [leucine-rich repeat]-, and PYD [pyrin domain]-containing protein 3) inflammasome. This inflammasome mediates the activation of IL-1β[26,27]. Colchicine has been indicated to prevent mitochondrial transport via microtubules, which impedes approximation of NLRP3, the sensor, to ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain (CARD))[26], the adaptor, and thus NLRP3 inflammasome assembly[28]. Direct NLRP3 inflammasome inhibitors do exist, such as small molecule MCC950, but this inhibitor is not further clinically developed due to liver toxicity during a Phase 2 rheumatoid arthritis trial[29]. Therefore, more specific targeting of the NLRP3 inflammasome in specific cells at play in atherosclerosis is a route of interest for drug development.

Atherosclerosis

The arterial wall is composed of multiple layers, which consists of a layer of endothelial cells in direct contact with the circulation, which are connected by adherens and tight junctions, and a subendothelial layer. This intima is followed by a medial layer consisting of smooth muscle cells, the adventitia and perivascular adipose tissue (PVAT). The endothelial monolayer of the intima becomes more permeable and activated during the initiation of atherosclerosis in areas of arteries with disturbed laminar flow. The expression of surface adhesion molecules such as vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) is increased, leading to recruitment of immune cells such as neutrophils and monocytes, of which the latter locally differentiate into macrophages[2,30–37]. Low and oscillatory shear stress can also cause upregulation of chemokines[38]. TNF-α increases chemokine CX3CL1 expression on endothelial cells under low shear stress conditions and, upon TNF-α stimulation and under low shear stress, CX3CL1 mediates the adhesion of monocytes to endothelial cells *in vitro*[39]. Moreover, low shear stress upregulates CX3CL1 *in vivo* and anti-CX3CL1 antibody treatment decreases the number of adventitial mast cells and intimal macrophages in low shear stress induced plaques[38]. Platelets can bind to the activated endothelial cells[40–42] and may subsequently recruit other cells[40,43,44]. Lipoproteins, such as low-density lipoprotein (LDL) with apolipoprotein B, can enter the subendothelial space via the permeable endothelial

layer[2,45]. There, the LDLs are modified by for example oxidation[46], which can form the signals that induce innate immune responses, such as cytokine and chemokine release from macrophages[2,47–51]. Neutrophils can in addition form neutrophil extracellular traps (NETs), which are DNA structures with granule and nuclear components[2,47,52,53]. A cascade of immune cell recruitment is induced, comprising B and T cells, which are cells of the adaptive immune system[2,54–61]. Additionally, vascular smooth muscle cells and fibroblasts located in the media start to move towards the endothelial layer and form a fibrous cap[2,62,63]. These cells also add to the fibrous cap by producing extracellular matrix components like collagen[2,62,63]. Intimal lipoproteins can be taken up by macrophages and smooth muscle cells. Their cholesteryl esters are hydrolyzed, with cholesteryl ester hydrolase or lysosomal acid lipase into free cholesterol and the free cholesterol is re-esterified. This is a continuous cycle called the cholesteryl ester cycle. Cholesterol is secreted as free/unesterified cholesterol and the hydrolysis is a rate limiting step in the efflux. When there is a disbalance between the uptake and efflux, cholesteryl esters will accumulate in the form of lipid droplets, forming foam cells[64–68]. The accumulating lipids in the foam cells induce endoplasmic reticulum stress and cell death which stimulates the formation of a necrotic core[2,69–71]. The dying foam cells release their lipids thereby contributing to the formation of the lipid pool. These extracellular lipid pools, which may also contain cholesterol-rich LDL, can be a location for crystal formation. Crystals are indicated to form in a mixture with phospholipids and cholesteryl esters. These crystals can also be formed upon cholesterol enrichment in the plasma membrane leading to the formation of cholesterol domains, which can function as nucleation site for crystal formation. Additionally, crystals can be formed in the lysosomes of cholesterol-loaded cells. The cholesterol crystals contribute to the necrotic core[72]. Additionally, oxidized LDL can be intracellularly transformed into cholesterol crystals and activate the NLRP3 inflammasome in macrophages, releasing the pro-inflammatory cytokine IL-1β[49,73].

Antigen presenting cells, for example dendritic cells (DCs), present antigens to T cells, which leads to T cell activation and expansion, but also antibody production by B cells[2,74–76]. During more advanced atherosclerosis, the production of various enzymes, such as serine proteases, matrix metalloproteinases (MMPs) and cathepsins by macrophages and mast cells, can lead to degradation of the extracellular matrix (ECM), which is suggested to contribute to plaque rupture[77–80]. Plaque rupture leads to thrombogenic components of the plaque being in contact with blood. This induces platelet binding to collagen components in the plaque and von Willebrand factor (vWF). The platelets become activated and subsequently activate more platelets, which leads to aggregation of the platelets via fibrinogen and vWF binding. At the same time tissue factor (TF), a protein expressed in the plaque by macrophage foam cells, vascular smooth muscle cells and their extracellular vesicles, is brought into contact with blood, inducing the coagulation cascade. This leads to thrombin formation, which in turn transforms fibrinogen into fibrin that activates platelets. The platelet-fibrin clot that is then formed is stabilized through fibrin crosslinking by activated factor XIII. The thrombus can occlude the artery and result in for example a myocardial infarction[2,81,82].

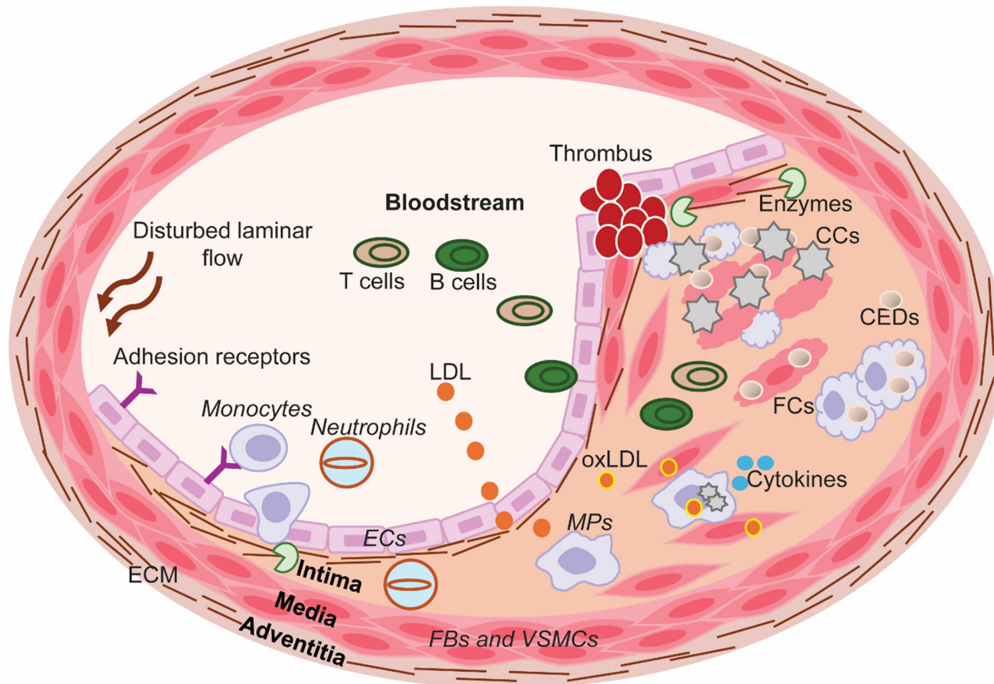

Figure 1. Atherosclerosis development, from initiation (left) to rupture (right). A disturbed laminar blood flow activates the endothelial cell (EC) monolayer of the intima and increases the expression of adhesion receptors, which leads to recruitment of immune cells, including the innate immune cells monocytes and neutrophils. The monolayer becomes permeable for low-density lipoprotein (LDL). LDL that migrates into the subendothelial space becomes modified, by for example oxidization. Modified LDL generates immune responses such as cytokine and chemokine production from innate immune cells, thereby inducing an immune cell recruitment cascade, which includes the adaptive T and B cells. Furthermore, vascular smooth muscle cells (VSMCs) and fibroblasts (FBs) migrate to the endothelial monolayer and produce extracellular matrix (ECM) components to form a fibrous cap. Smooth muscle cells (SMCs) and macrophages (MPs) can form foam cells (FCs) with cholesteryl ester droplets (CEDs) when there is a disbalance between lipid uptake and efflux of unesterified cholesterol. The foam cells will die and form a necrotic core. Cholesterol crystal (CC) formation adds to the core. Enzymes, including proteases, are able to degrade the ECM, enhancing the risk of plaque rupture. Upon rupture a thrombus can be formed through platelet activation and initiation of the coagulation cascade.

Immune cells in atherosclerosis

The immune system, which responds to infections and endogenous abnormalities, consists of two main parts, the innate and adaptive immune system[83]. First, the innate immune system responds to an infection, after which the antigen-presenting cells activate the adaptive immune system. This adaptive system is able to form immune memory[84]. All immune cells originate from hematopoietic stem cells (HSCs) residing in the bone marrow. In the classical hematopoietic hierarchy, HSCs give rise to multipotent progenitor cells (MPPs). MPPs subsequently differentiate into two lines of common progenitors: common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs). CMPs give rise to megakaryocyte-erythrocyte progenitors (MEPs) and granulocyte-monocyte precursors (GMPs). MEPs form

megakaryocytes and erythrocytes via their precursors. GMPs form granulocytes (neutrophils, basophils, eosinophils and mast cells[85]), myeloid dendritic cells (mDCs) and monocytes. Monocytes can subsequently differentiate into macrophages[37,86]. The CLPs give rise to T, B and NK cells via their pro-cells[86]. In the recent years, subsets of all these cell types have been identified, such as different macrophage subsets, using single-cell technologies, which will be discussed in this thesis.

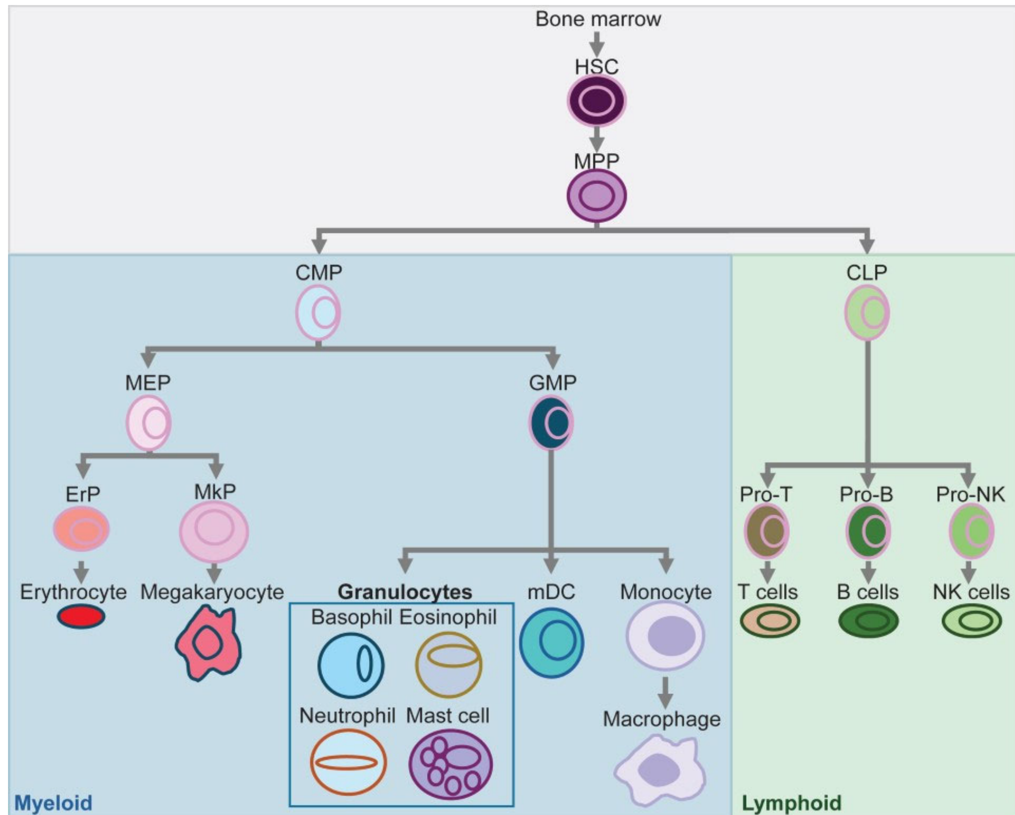

Figure 2. Classical hematopoietic hierarchy. Hematopoietic stem cells (HSCs) are located in the bone marrow and give rise to common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs) via multipotent progenitors (MPPs). CMPs first form megakaryocyte-erythrocyte progenitors (MEPs) and granulocyte-monocyte precursors (GMPs) and MEPs subsequently form megakaryocytes and erythrocytes precursors which give rise to megakaryocytes and erythrocytes. GMPs give rise to granulocytes, including neutrophils, basophils, eosinophils and mast cells, but also myeloid dendritic cells (mDCs) and monocytes. Next, monocytes form macrophages. The CLPs give rise to pro-T, -B and -NK cells and these subsequently form T, B and NK cells.

Innate immune cells are generally of the myeloid lineage, such as the neutrophils, basophils, eosinophils, mast cells, monocytes, macrophages and DCs. Also the NK cell, a lymphocyte, is of the innate immune cell class[84]. Innate immune cells recognize common pathogen and damage-associated molecules via pattern recognition receptors (PRRs)[87,88] to generate an anti-inflammatory response. Five PRR classes exist: Toll-like receptors (TLRs), nucleotide oligomerization domain (NOD)-like receptors (NLRs), C-type lectin receptors (CLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) and absent in melanoma-2 (AIM2)-like

receptors (ALRs). The common molecules recognized by these receptors can be pathogen-associated molecular patterns (PAMPs), which are specific molecules that particular pathogens share and do not exist in the cells of the host, such as bacterial lipopolysaccharides, which signals via TLR4, and bacterial double-stranded DNA via ALRs[88]. PRRs can also recognize damage-associated molecular patterns (DAMPs), which are from the host itself and are released from a cell upon for example necrosis[88]. An example is oxidized LDL that can signal via TLR4 and TLR6[51].

The lymphoid T and B cells belong to the adaptive immune cell populations[84]. Adaptive immune cells have antigen receptors which can be activated by antigens presented by antigen-presenting cells, such as DCs and macrophages. This activation will lead to differentiation of naïve adaptive cells into effector cells and also memory cells can be formed. Upon re-exposure to the antigen the memory cells can differentiate into effector cells and form an instant response[84]. Furthermore, data indicate a role for antigen presentation in atherosclerosis by the B2 B cell subset to CD4 T cells[89]. Another example of a specific B cell subset involved in atherosclerosis is the age-associated B cell subset, which is shown to be enriched for an antigen presentation associated signaling pathway in the atherosclerotic aortas of *Ldlr*$^{-/-}$ mice[2,90].

In this thesis, we will focus mostly on specific myeloid populations which are described below in more detail.

Monocytes

Monocytes are hematopoietic innate immune cells that are recruited to the atherosclerotic plaque from the circulation via chemokine receptors and adhesion molecules[33,37,91–95]. In the plaque, macrophage-colony-stimulating factor (M-CSF) mediates monocyte differentiation into macrophages[37,94,96]. The three circulating human monocyte subtypes are classical monocytes ($CD14^{++}CD16^{-}$), intermediate monocytes ($CD14^{++}CD16^{+}$) and non-classical monocytes ($CD14^{+}CD16^{++}$)[97]. In mice, monocytes are generally subdivided in two major subsets, namely the classical ($Ly6C^{hi}$) and non-classical ($Ly6C^{lo}$) monocytes[93,94]. Human classical monocytes have been shown to secrete more of the pro-inflammatory cytokine IL-1β upon bacterial LPS and Flagellin signaling and more IL-6 upon Flagellin signaling than the intermediate and non-classical monocytes[98]. Non-classical monocytes seem to patrol the endothelium of the vasculature and increase the expression of E-selectin on endothelial cells by secreting TNF-a, which induces the recruitment of neutrophils[94,99,100]. In addition to classical and non-classical monocytes, more recently the following human monocyte subsets have been identified: interferon-responsive, major histocompatibility complex class II^{hi} ($MHC\text{-}II^{hi}$) and monocyte-platelet aggregates. Bashore et al. show an increase in interferon-responsive and non-classical monocytes in individuals with hyperlipidemia (low-density lipoprotein cholesterol (LDL-C) higher than 130 mg/dL) compared to individuals with normal LDL-C (lower than 100mg/dL), which may suggest a link between these monocyte subsets and CVDs[101].

Macrophages

Macrophages in the atherosclerotic plaque can be derived from monocytes that have been recruited to the plaque[33,37,92,93], while macrophages have also been shown to originate from locally proliferating macrophages[102]. Previously, macrophages were divided into either M1 or M2 macrophages. In the recent years however, a number of more specific macrophage subsets in human and mouse plaques were identified using single-cell techniques. Fernandez et al. for example identified two macrophage subsets in the human plaque, namely CD64^{+}HLADR^{+}CD206^{hi} and CD64^{+}HLADR^{+}CD206^{lo}, based on the expression of the M2 marker CD206[103]. Depuydt, Prange and Slenders et al. later on presented three macrophage subsets in human plaques, *IL1B*$^{+}$ inflammatory macrophages, *TNF*$^{+}$ inflammatory macrophages and *ABCG1*$^{+}$ foamy macrophages[104]. Zernecke et al. found three major macrophage subsets in human atherosclerosis hInflammatory (*CD74, HLA-DRB1*), hFoamy (*APOC1, APOE, FABP5, FABP4*) and hLYVE1 (*LYVE1, LGMN, MARCO*) macrophages, but also two smaller subsets, hC3, which express *C3*, *JUN* and *CCL4* and hIFNIC macrophages, also named type I interferon response macrophages[105]. These authors also integrated single-cell RNA-sequencing datasets of mouse atherosclerotic aortas with human data and found four integrated macrophage clusters, which are defined as Resident/Resident-like, Foamy/TREM2^{hi}, IFNIC and inflammatory macrophages[105], similar clusters are presented by Cochain et al. obtained from mouse data[106]. Mosquera et al. have used four existing human atherosclerotic single-cell studies and identified inflammatory, two foamy, tissue resident and phagocytosis macrophages[107]. The dichotomy between lipid-associated (TREM2^{hi}) and inflammatory macrophages appears to be less rigid after the identification of PLIN2^{hi}/TREM1^{hi} macrophages in human plaques by Dib et al., which have an inflammatory lipid-associated signature[108]. Zernecke et al. also identified various additional clusters in the mouse datasets. The resident macrophages consist of two clusters, *Cd209*^{low} and *Cd209*^{hi}. The inflammatory subset apparently consists of two clusters, the first being inflammatory-*Nlrp3* macrophages which have high *Nlrp3* and *Il1b* expression and the second subset being CCR2^{Int}MHCII^{+} macrophages, which display intermediate *Ccr2* and *Cd74*, *H2-Aa*, *H2-Eb1* expression. Additionally, the foamy macrophages also contain two clusters, *Trem2*^{hi}-*Slamf9* and *Trem2*^{hi}-*Gpnmb*. Furthermore, additional clusters were identified, including small peritoneal macrophages (SPMs), interferon inducible cells (IFNIC) and intimal resident macrophages (Mac-AIR)[105]. Cole et al. described five macrophage subsets in the aorta of *apoE*$^{-/-}$ mice, which include CD206^{+}CD169^{+}CD209b^{+}, CD206^{+}CD169^{+}CD209b^{-}, CD11c^{+}, CD206^{lo-int} and F4/80^{hi}CD11b^{hi} macrophages[109]. Lastly, Winkels et al. show 2 macrophage subsets in the aorta of *apoE*$^{-/-}$ mice, *Lyve1*^{low}*Ly6c1*^{neg} and *Lyve1*^{neg}*Ly6c1*$^{+}$[110]. Summarizing, the macrophage population in the atherosclerotic plaque is quite heterogeneous and consists of multiple subsets identified by specific gene signatures, which may overlap between studies. A pro-inflammatory subset expressing *NLRP3* is clearly established in multiple studies. The NLRP3 inflammasome is indicated to be a key player in atherosclerosis. Inflammasome proteins are mainly expressed in macrophages and foam cells in human carotid plaques[111,112]. Expression of *NLRP3* mRNA is higher in symptomatic than asymptomatic human carotid plaques[111], and NLRP3 protein expression correlates with the severity of coronary artery disease[113]. The NLRP3 inflammasome is a supramolecular multiprotein complex located inside the cell[73,114]. It is build up out of three types of proteins, NLRP3, the sensor, ASC, the adaptor, and pro-caspase-1, the effector[27,73]. NLRP3 contains a C-terminal leucine-rich repeat (LRR), a central nucleotide-binding and oligomerization domain (NOD or NACHT) and a N-terminal pyrin domain (PYD)[73,115]. ASC also contains a N-terminal PYD and a C-terminal caspase recruitment domain

(CARD)[73,116]. The enzyme pro-caspase-1 also contains a CARD, on the N-terminus, followed by a p20 subunit and a C-terminal p10 subunit[73,117]. For NLRP3 inflammasome activation and pro-caspase-1 cleavage, the NLRP3 inflammasome has to be assembled, for which different signals are required[73]. First, priming with for example LPS occurs through TLRs or cytokine receptors, via nuclear factor-κB, thereby inducing NLRP3 and pro-IL-1β protein[73,118]. Also, post-translational modification (PTM) of the NLRP3 protein regulates priming[73,119] (Figure 3). After priming, an activation signal, induced by for example cholesterol crystals in atherosclerosis, and via lysosomal damage and cathepsin release[73,120,121], leads to NLRP3 inflammasome assembly (Figure 3). Upon NLRP3 activation, PYD-PYD interactions with ASC are formed, which nucleates the PYD filaments of ASC. Subsequently ASC forms CARD-CARD interactions with pro-caspase-1 and nucleates the caspase-1 CARD filaments, which activates caspase-1[114]. Caspase-1 is a protease and cleaves pro-IL-1β and pro-IL-18 into their active forms. Additionally, caspase-1 cleaves gasdermin D (GSDMD) and the released N-terminal domain (GSDMD-N) makes oligomeric pores. The pro-inflammatory cytokines IL-1β and IL-18 are released through these pores, inducing an inflammatory response and pyroptosis[73,117,122–129].

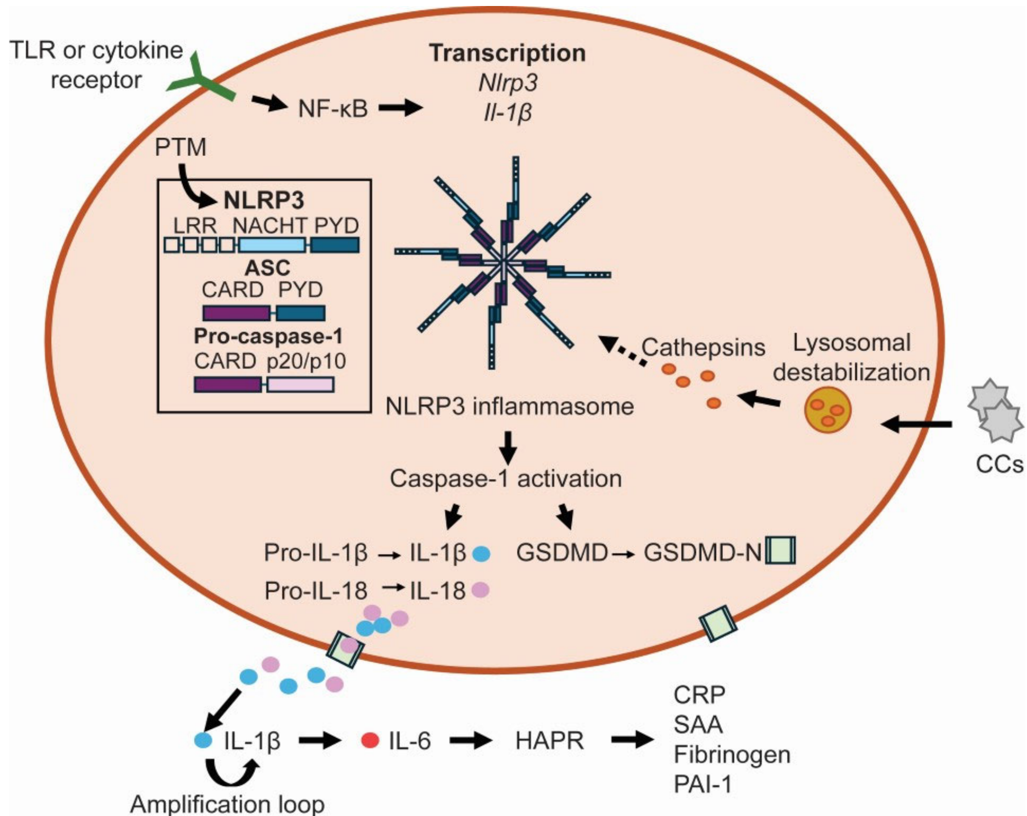

Figure 3. NLRP3 (NOD [nucleotide oligomerization domain]-, LRR [leucine-rich repeat]-, and PYD [pyrin domain]-containing protein 3) inflammasome activation mechanisms. The inflammasome consists of three types of proteins, the sensor protein NLRP3 (leucine-rich repeat (LRR), nucleotide-binding and oligomerization (NACHT or NOD) domain and pyrin domain (PYD)), the adaptor protein ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain

(CARD)) (two domains, PYD and CARD), the effector pro-caspase-1 (domain CARD and the two subunits p20 and p10). To activate the NLRP3 inflammasome a priming and an activation signal are needed. A priming signal through Toll-like receptors (TLRs) or cytokine receptors via nuclear factor-κB (NF-κB) induces NLRP3 and pro-IL-1β protein production. Additionally, post-translation modification (PTM) of NLRP3 regulates priming. Activation signals, for example cholesterol crystals (CCs) via lysosome destabilization and cathepsins, induce assembly of the NLRP3 inflammasome. Subsequently, caspase-1 produces the active IL-1β and IL-18 and cleaves gasdermin D (GSDMD). The N-terminal domain (GSDMD-N) forms pores, enabling pro-inflammatory cytokine release and pyroptosis. IL-1β can amplify itself and can induce IL-6, which can lead to a hepatocyte acute phase response (HAPR), including production of C-reactive protein (CRP), serum amyloid A (SAA), fibrinogen and plasminogen activator inhibitor-I (PAI-I).

The produced IL-1β can signal via the IL-1 receptor type 1 (IL-1R1)[130], which is also an optional priming signal for the NLRP3 inflammasome[131,132]. Thus, in these cells IL-1β can induce an amplification loop of its own production[130,133] (Figure 3). The next step in the NLRP3 inflammasome-IL-1β pathway is the induction of IL-6 by IL-1β[134], which can subsequently induce an acute phase response in the liver. This response includes the formation of serum amyloid A, the inflammation biomarker C-reactive protein, the thrombin precursor fibrinogen and the inhibitor of the endogenous fibrinolytic system, plasminogen activator inhibitor-1 (Figure 3)[135,136].

Foamy macrophages are another set of macrophages present in atherosclerotic plaques[137]. Foam cell death and impaired efferocytosis, defined as impaired clearance of the apoptotic cells, by macrophages in advanced plaques, contribute to necrotic core formation[138,139]. Interestingly, the NLRP3 inflammasome seems to be able to affect efferocytosis. This is demonstrated by an increase in efferocytosis capacity by macrophages upon caspase-1 inhibition in *apoE$^{-/-}$* mice, but not in *apoE$^{-/-}$Nlrp3$^{-/-}$* mice[140].

In atherosclerotic plaques, macrophages are the main producers of proteases[141–143] such as MMPs[144], cathepsins[145] and serine proteases[142], which are, as mentioned before, able to induce ECM degradation, thereby contributing to plaque rupture[77–80,146,147]. Proteases, also called peptidases, are peptide-bond cleaving enzymes. These enzymes can be grouped into endopeptidases and exopeptidases, where endopeptidases cleave the target protein at the inner region of the peptide chain and exopeptidases cleave in the ends or close to the ends of the chain. The main proteases that degrade ECM proteins are the endopeptidases. The endopeptidases that function extracellularly are metalloproteinases (which include MMPs), serine peptidases and cysteine endopeptidases (which include many cathepsins)[143]. The ECM in the plaque contains proteins such as collagen, proteoglycans, elastin, fibronectin and laminin[148]. About 60% of for example calcified plaques consists of collagen[149]. Collagen can be degraded by MMPs, of which multiple classes exist, such as collagenases, gelatinases and stromelysins. Collagenases 1,2 and 3 (MMP1, 8 and 13) and gelatinase A (MMP2) can degrade collagens and stromelysin-1 (MMP3) can degrade collagen telopeptides. Other examples of substrates for these classes are gelatin for MMP1, 8, 13, 2 and 9 (Gelatinase B), fibronectin for MMP1, 13, 9, 3, 10 (Stromelysin-2), 11 (Stromelysin-3)[150]. In human atherosclerotic plaques, MMPs have been found to colocalize with macrophages[146,147,151–156]. Serine peptidases include for example neutrophil elastase, cathepsin G, chymase and tryptase[143]. Neutrophil elastase is able to degrade elastin, fibronectin, laminin, proteoglycans and type III, IV and VI collagen[157]. Neutrophil elastase, is a serine protease that has been shown to colocalize with macrophages in vulnerable human plaques[142]. Lastly, the cysteine proteases include both enzymes related to

IL-1β-converting enzyme and the papain superfamily of cysteine proteases, which includes the cathepsin family, including for example cathepsin B, S and K[143]. Cathepsin activity was also shown to be higher in CD206$^+$ macrophages in unstable plaques than in stable plaques. This suggests that mainly these macrophages increase the cathepsin activity in unstable plaques and contribute to plaque rupture[145].

Mast cells

Mast cells have been identified for the first time by Paul Ehrlich in 1878[158], as granular cells in connective tissue using aniline dyes. Mast cells derive from hematopoietic stem cells in the bone marrow. First these stem cells give rise to mast cell progenitors that circulate in the blood and these progenitors differentiate into mature mast cells upon tissue entrance[85,159]. Mast cells are characterized by their expression of both c-kit/CD117 and the Fc epsilon receptor 1 (FcεRI), which is the high affinity IgE receptor[160]. Currently, mast cells are divided into two subtypes in humans, based on their protease content, mast cells containing tryptase (MC_T) and mast cells containing both tryptase and chymase (MC_{TC})[159,161–163]. In mice, also two mast cell subsets have been described, connective tissue mast cells and mucosal mast cells. The major mouse proteases of mast cells are mast cell protease 1, 2, 3, 4 (chymase), 5, 6 (tryptase), 7 (tryptase) and carboxypeptidases. Mast cell protease 4 (chymase), 5, 6 (tryptase) and carboxypeptidase A3 are expressed more by connective tissue mast cells, while mast cell protease 1 and 2 are expressed more by mucosal mast cells, but their protease phenotypes seem tissue regulated[163,164]. The proteases are stored in granules together with histamines and proteoglycans (PGs) with side chains of heparin (HEP) or chondroitin sulfate[165–168]. Upon sensitization of mast cells with IgE via the FcεRI, antigens can bind to crosslink the mast cell-bound IgE, leading to mast cell degranulation and release of granules in its surroundings[161]. In addition to the FcεRI, mast cell express a wide variety of receptors, such as complement receptors[169] and the TLR4 receptor to which oxidized LDL and bacterial components can bind[170]. In human atherosclerotic plaques, mast cells have been indicated to accumulate[171,172] and their number in the plaque associates with future cardiovascular events and microvessel density[172]. Mast cells can secrete the angiogenic mediator basic fibroblast growth factor (bFGF) in human atherosclerosis and bFGF$^+$ mast cells associated with the number of microvessels in the plaque and atherosclerosis severity[173]. These studies suggest that mast cells can contribute to plaque progression via the formation of these microvessels. Additionally, mast cells may contribute to atherosclerosis via the release of chymase and tryptase[162]. Inhibition of chymase inhibited plaque destabilization in *apoE*$^{-/-}$ mice[174]. Also, chymase and tryptase can activate MMPs[175]. Furthermore, mast cells may contribute via HEP-PGs, which can form complexes with LDL and stimulate macrophages to engulf the LDL[176]. However, mast cell HEP-PGs can also exert antithrombotic effects as these PGs have been shown to inhibit platelet aggregation by collagen *in vitro*[177]. In addition, HEP-PGs inhibit platelet activation and deposition on collagen in a flowing blood model[178]. To conclude, mast cells can contribute to atherosclerosis progression via a variety of mechanisms, but certain mast cell components can have protective effects as well.

Dendritic cells

DCs are another set of hematopoietic innate immune cells[179] and known for their ability to present antigens on their surface MHC-proteins to T cells. In general, DCs are classified in two main classes: conventional DCs and plasmacytoid DCs. The plasmacytoid DCs contain large quantities of interferons, which can induce various T cell responses, such as cytotoxic $CD8^+$ T cell expansion and $CD4^+$ T cell to T-helper 1 polarization. Plasmacytoid DCs can also present antigens via MHC-II to $CD4^+$ T cells. The conventional DCs can be divided into type 1 and type 2 conventional DCs. Type 1 conventional DCs cross-present exogenous antigens via MHC-I to $CD8^+$ T cells. Type 2 conventional DCs play a role in T cell response initiation[180]. Single-cell techniques, such as cytometry by time of flight (CyTOF) and single-cell RNA-sequencing, enabled the identification of human plaque DC clusters. While Depuydt, Prange and Slenders et al. identified one DC population, the $CD1c^+$ population[104], Fernandez et al. identified both plasmacytoid DCs and $CD1c^+$ DCs[103]. Bashore et al. revealed the presence of plasmacytoid DCs and $CD141^+$ conventional DCs in human atherosclerosis[181]. In atherosclerotic aorta's of mice, Cole et al. and Winkels et al. have found three DC clusters[109,110], which have been identified as type 1 conventional DCs, type 2 conventional DCs and plasmacytoid DCs by Cole et al.[109]. Later, Zernecke et al. classified DCs in mouse atherosclerosis into the following three subsets: type 1 classical DCs, DCs derived from monocytes and/or type 2 classical DCs and mature DCs. The mature DCs have a more immunoregulatory signature[105]. In 1997, foam cells in human atherosclerotic lesion have been shown to have DC characteristics[182]. Furthermore, in 2010, Paulson et al. show that during atherosclerosis development in *Ldlr*$^{-/-}$ mice, foam cells in aortic lesions express DC markers and that DC depletion reduces the lipid area in aortic lesions, showing a pro-atherogenic role for DCs[183]. Interestingly, depletion of plasmacytoid DCs by Daissormont et al. worsened lesion development and progression in *Ldlr*$^{-/-}$ mice, indicating a protective function in atherosclerosis for plasmacytoid DCs[184].

Thesis outline

Myeloid cells can play a crucial role in atherosclerosis via for example cytokine secretion and protease release. These proteases, including serine proteases, MMPs and cathepsins, can degrade the ECM which can lead to plaque rupture and subsequent acute cardiovascular events. In **Chapter 2** we study the expression of serine proteases in advanced human atherosclerotic plaques by analyzing the presence of proteases of the serine hydrolase enzyme class. Activity-based protein profiling (ABPP) is used to identify the active serine hydrolases in plaques from carotid endarterectomy patients. In addition, single-cell RNA-sequencing datasets were analyzed to determine which cell subsets express these enzymes. The mast cell is an immune cell known for its protease content. The proteases are stored in granules together with proteoglycans like heparin proteoglycans (HEP-PGs). Mast cell HEP-PGs can have antithrombotic effects, but may also have anti-inflammatory properties[185]. As both inflammatory as well as thrombotic factors are known to contribute to atherosclerotic development as well as to thrombus formation upon plaque rupture[42,186], the HEP-PGs can be of interest for atherosclerosis therapy development. Hence, in **Chapter 3** the effect of a HEP-PG bioconjugate with antiplatelet and anticoagulant activity, called APAC, is studied in an *in vivo* atherosclerosis model.

The second part of this thesis is focused on the NLRP3 inflammasome and related cytokines in preclinical atherosclerosis, and more specifically on the pro-inflammatory cytokines IL-1β and IL-18, which are secreted mainly by myeloid cell subsets upon activation of the NLRP3 inflammasome. Interestingly, a subset of the human atherosclerotic plaque myeloid cells co-express the IL-1R1, via which IL-1β can signal, and NLRP3. Therefore, in **Chapter 4,** a new bispecific antibody called InflamAb, which targets IL-1R1^{+}NLRP3^{+} cells, is investigated for its atherosclerosis inhibition potential. Potential therapeutics targeting these cytokines are mainly tested in mouse models. The translation to human disease can be impeded by the study set-up, which includes animal sex. Therefore, we investigated the therapeutic efficacy of IL-1, IL-18 and IL-6 signaling interventions in male and female animals in experimental models of atherosclerosis in **Chapter 5**.

In **Chapter 6**, a summary and discussion of the studies described in this thesis and future perspectives are provided.

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