Disarray at the membrane

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Regulation of vascular inflammation
by cholesterol and proteases

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Disarray at the membrane

Regulation of vascular inflammation
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Chapter *1*

General introduction

Atherosclerosis

Cardiovascular diseases (CVDs) are the leading cause of morbidity and mortality worldwide ¹. The underlying pathology of the majority of cardiovascular-related deaths is atherosclerosis, which is a progressive disease characterized by the accumulation of lipids, inflammatory cells and fibrous tissue within the vessel wall. Epidemiological studies have revealed several important risk factors associated with atherosclerosis, including age, hypertension, tobacco use, stress, physical inactivity and dyslipidemia ². However, mechanisms of the atherosclerotic process have been proven difficult to assess in humans, due to the chronic and multifactorial nature of the disease, as well as the inability to monitor atherosclerotic lesion progression in an individual in a non-invasive manner. Using descriptive pathology of human autopsies, Stary *et al.* ³ and Virmani *et al.* ⁴ have histologically identified and classified several types of atherosclerotic lesions. However, our biological understanding of the mechanisms underlying atherosclerosis development stems mainly from animal models with severe hypercholesterolemia, especially mouse models, in which modulation of atherosclerosis development through genetic alterations is highly similar to gene-phenotype associations found in humans ⁵. Intensive study of the pathophysiology of the atherogenic process has led to the following consensus view (outlined in **figure 1**).

The process of atherosclerosis is initiated by the subendothelial retention of plasma apolipoprotein B (apoB)-containing lipoproteins, such as low-density lipoprotein (LDL), in medium- and large-sized arteries, particularly at regions of disturbed laminar blood flow ⁶. Components of LDL can become biochemically modified (e.g. oxidized), which trigger an inflammatory response in the vessel wall. Activated endothelial cells upregulate their expression of adhesion molecules and chemokines, thereby attracting leukocytes towards the site of inflammation. Recruited monocytes differentiate into macrophages, which scavenge modified LDL and further aggravate the inflammatory reaction ⁷. The initial lesion is characterized by the accumulation of lipid-laden macrophages, called foam cells, and extracellular lipids. As cellular stress increases due to various agents, including lipids, cytokines and reactive oxygen species, (lipid-laden) macrophages become apoptotic, which, in initial lesions, are effectively cleared by surrounding macrophages in a process termed efferocytosis ⁸. In more advanced lesions, however, apoptotic cells coalesce into a lipid-rich necrotic core due to an increased rate of apoptosis and/or defective clearance. Furthermore, activated vascular smooth muscle cells (VSMCs) switch from a quiescent contractile to a synthetic phenotype, which can proliferate and migrate into the lesion ⁹. In addition, they increase their production of extracellular matrix components (e.g. collagen and proteoglycans), forming a fibrous cap overlying the necrotic core, which provides a protective barrier between coagulation factors in the blood stream and pro-thrombotic material in the plaque. In contrast, rupture prone atherosclerotic lesions are associated with the presence of intraplaque neovascularization and haemorrhage ¹⁰. Intraplaque microvessels fuel the atherosclerotic process by providing additional entry points for lipids and leukocytes into the atherosclerotic lesion ¹¹. Additionally, these microvessels often lack a continuous basement membrane and mural cell coverage, and have poorly formed tight junctions, which makes them highly susceptible to rupture and intraplaque haemorrhage, resulting in the spillage of cholesterol-rich erythrocytes, lipids

and leukocytes into the lesion¹². Plaque destabilization, through thinning of the fibrous cap by matrix degrading metalloproteinases and VSMC death, together with necrotic core expansion, neovascularization, intraplaque hemorrhage and ongoing inflammation, leads to plaque rupture¹³. The resulting thrombus formation may cause occlusion of the vessel leading to clinical manifestations, like myocardial infarction or stroke.

Figure 1: Schematic representation of atherosclerosis development. Early atherosclerosis development is characterized by the accumulation of lipids (e.g. low density lipoprotein; LDL) in the vessel wall. Activation of endothelial cells leads to the active recruitment of leukocytes, including monocytes, which differentiate into macrophages and scavenge (modified) lipids (e.g. oxidized LDL; oxLDL), yielding foam cells. During lesion progression, smooth muscle cells migrate into the intima and secrete extracellular matrix components, which form the fibrous cap. Cells within the plaque, including foam cells, die which eventually leads to formation of a lipid-rich necrotic core. In addition, neovascularization can accelerate plaque growth by providing an additional entry point of leukocytes and plasma constituents, and via intraplaque hemorrhage. Ultimately, disruption of the fibrous cap by smooth muscle cell death and matrix degrading metalloproteinases can lead to plaque rupture, acute thrombosis and clinical events (adapted from Ruparelia *et al.*¹⁴).

High-density lipoproteins: more than just carriers of cholesterol

Cholesterol has a fundamental role in mammalian physiology by modulating cell membrane fluidity and signaling, as well as serving as a precursor to steroid hormones, oxysterols and bile acids¹⁵. Maintaining cholesterol homeostasis, both at a cellular, as well as on whole body level, is essential to mammalian health. However, hypercholesterolemia, i.e. high plasma levels of cholesterol, can trigger inappropriate immune responses and the development of atherosclerosis and thereby represents a major risk factor for CVD¹⁶.

Cholesterol originates from *de novo* synthesis and dietary intake. Because of its hydrophobic characteristics, cholesterol is packaged and distributed to most tissues via the circulation within lipoprotein particles. These particles are heterogeneous in size, density, composition and function (extensively reviewed by Ramasamy¹⁷). Initially, dietary cholesterol absorbed by the small intestine is packaged, along with triglycerides, into apoB-48 containing chylomicrons. Upon entrance of the circulation, triglycerides in chylomicrons are hydrolyzed by lipoprotein lipase, thereby generating chylomicron remnants, which are taken up by hepatocytes. In turn, the liver secretes cholesterol in apoB-100 containing very-low density lipoprotein (VLDL) that are processed in the circulation into LDL, the main lipoprotein that delivers cholesterol to peripheral tissues. In contrast, apolipoprotein A-I (apoA-I) containing high-density lipoproteins (HDL) shuttle excess cholesterol from peripheral tissues back to the liver for excretion into the feces in the form of neutral sterols or bile acids, in a process termed reverse cholesterol transport (RCT)¹⁸. ApoA-I, which is synthesized and secreted by the liver and intestine, comprises ~80% of the total HDL proteome^{18,19}. Binding of lipid-free apoA-I to the ABC transporter A1 (ABCA1) triggers a multi-step process by which cholesterol and phospholipids are transferred to apoA-I, thereby generating HDL. Additional cholesterol extraction from peripheral cells via ABCA1, ABC transporter G1 (ABCG1) or scavenger receptor B1 (SR-B1) results in the further maturation of HDL¹⁸.

Excess intake of cholesterol is associated with increased plasma levels of apoB-containing particles, including LDL, which are directly related to the CVD risk¹⁷. Currently available therapeutics for treatment of atherosclerosis are largely restricted to alleviating hypercholesterolemia⁶. HMG-CoA reductase inhibitors, also known as statins, have been successful in reducing the incidence of CVD events by lowering the plasma levels of atherogenic lipoprotein particles. However, despite the effectiveness of statins in lowering LDL cholesterol and preventing CVD events, there still remains a residual risk of recurrent CVD events, even after aggressive/intensive statin therapy²⁰. Therefore, research has been redirected towards additional novel therapies, including more potent LDL cholesterol lowering therapies (e.g. proprotein convertase subtilisin/kexin type 9; PCSK9 inhibitors)²¹, immunomodulatory strategies (e.g. interleukin 1 neutralizing antibodies)²² and HDL raising therapies²³.

In contrast to LDL, findings of epidemiological studies showed that plasma levels of HDL cholesterol (HDL-C) are strongly inversely associated with the incidence of

cardiovascular events²⁴⁻²⁶. In addition, preclinical studies using HDL infusions or overexpression of apoA-I, both of which increase plasma HDL-C levels, resulted in the inhibition or even regression of atherosclerotic lesions in mouse and rabbit models²⁷⁻³¹. HDL can provide protection against atherosclerosis in a myriad of ways. The most established anti-atherogenic property of HDL is its role in the RCT process, in which excess cholesterol is extracted from the artery wall and transported towards the liver for excretion³². In addition, a large body of data accumulated over the past few years has shown that HDL has potent immunomodulatory properties in a variety of cell types. By acting at the level of hematopoietic stem cells (HSCs), HDL can prevent the onset of myeloproliferative disorder, by suppressing HSC proliferation and the production of monocytes and neutrophils³³. Furthermore, HDL can attenuate leukocyte recruitment into arteries by reducing the expression of adhesion molecules and chemokines by endothelial cells³⁴⁻³⁶ and monocytes^{35,37}. Moreover, HDL suppresses dendritic cell (DC) activation, resulting in the failure to promote antigen-specific T helper cell proliferation³⁸ and their ability to polarize naïve T helper cells towards a Th1 phenotype³⁹. On the other hand, research on the effects of HDL on macrophage inflammatory responses is limited and has even yielded conflicting results, in which HDL has been shown to dampen⁴⁰ but also promote⁴¹ inflammatory responses in these innate immune cells, at least *in vitro*. Lastly, besides its role in the RCT process and inflammatory reactions, HDL also possess anti-oxidative, anti-thrombotic, and endotoxin-neutralizing activities, and can enhance endothelial (progenitor) cell function and repair (outlined in **figure 2**)⁴².

These observations instigated a large interest in HDL as an alternative therapeutic target for the treatment of CVD. However, recent clinical trials of HDL-raising therapies have led to great concerns about the causality of HDL in atherosclerosis⁴³. Several studies have challenged the concept that HDL-C levels are predictive of future CVD events. Human genome-wide association studies have identified many single-nucleotide polymorphisms (SNPs) which were associated with increased plasma levels of HDL-C, however, these SNPs did not correlate with any changes in risk of CVD⁴⁴⁻⁴⁶. In addition, post-translational modifications of HDL can render these particles dysfunctional, resulting in the loss of their cardioprotective properties^{47,48}. Furthermore, loss-of-function variants of the genes encoding for endothelial lipase and SR-B1, which lead to increased plasma HDL-C levels, are accompanied by an unchanged or even an increased risk of CVD events, respectively^{45,49}. Conversely, carriers of the apoA-I^{Milano} mutant have very low plasma levels of HDL without increased risk of clinical events⁵⁰.

Taken together, the current data suggest that the role of HDL in CVDs is more complicated than was previously thought. Further investigation in cell type-specific functions of HDL is essential for developing novel or improved therapeutic approaches to reduce the residual risk for CVD events using HDL.

Figure 2: High-density lipoprotein actions in various cell targets. ECM, extracellular matrix; HSC, hematopoietic stem cells; NO, nitric oxide; ROS, reactive oxygen species (adapted from Mineo *et al.* ⁴²).

Immunomodulation by post-translational proteolytic processing of transmembrane proteins

The characterization of atherosclerosis as an immune-mediated vascular disease, as well as the observation that dampening of the hyperactive immune response in animal models of atherosclerosis can inhibit atherosclerosis progression, has led to efforts to develop immunomodulatory strategies for the prevention and treatment of CVDs²². Proteolytic processing of transmembrane proteins is emerging as a novel level of control of many biological processes, including immune responses^{51,52}. In fact a wide variety of inflammatory mediators, including adhesion molecules, cytokines, chemokines and receptors can be subjected to functional modulation by limited proteolysis, an irreversible post-translational modification, in which proteins are functionally modified by a proteolytic cleavage yielding truncated, stable cleavage products⁵³. Two fundamentally different cleavage mechanisms are (ectodomain) shedding and regulated intramembrane proteolysis, which result in the release of the extracellular or intracellular domains of single span transmembrane proteins, respectively (outlined in **figure 3**).

Figure 3: Schematic representation of shedding and regulated intramembrane-proteolysis (RIPping). Shedding, by A Disintegrin And Metalloproteinases (ADAM) or any other protease with sheddase-activity, produces a soluble ectodomain and a membrane-bound stub, which can be further processed by an intramembrane-cleaving protease (e.g. SPPL), thereby generating an intracellular domain that is further degraded or can function as a signaling molecule (adapted from Gillian⁵⁴ and Schneppenheim *et al.*⁵⁵).

Through shedding, cells can rapidly reduce the expression of surface proteins, while simultaneously, producing soluble functional protein ectodomains that can function in an autocrine or paracrine manner. Once released, soluble ectodomains exhibit functions similar to or distinct from its cell surface bound variant⁵³, as exemplified by CX₃CL₁, which acts as an adhesion molecule in its membrane-bound form, while functioning as a chemokine upon shedding⁵⁶. Although many proteases possess the capacity to function as 'sheddas', one prominent group of proteases dedicated to shedding is the A Disintegrin And Metalloproteinases (ADAMs) family. ADAMs were originally discovered as transmembrane proteins implicated in fertilization, however a tremendous amount of progress has been made in the recent years uncovering many additional roles both in physiological as well as pathological processes, including inflammation⁵⁷.

The expression of one member of the ADAMs protease family, ADAM8, has consistently

been shown to be increased in many diseases with an inflammatory component, including asthma, cancer and atherosclerosis, although its precise role in these diseases is ill defined^{58,59}. Mice lacking ADAM8 do not show an overt phenotype, suggesting that ADAM8 is dispensable for embryonic and adult homeostasis⁶⁰, which makes ADAM8 an attractive therapeutic target.

In contrast, ADAM10 deficient mice are embryonically lethal and are characterized by defects in their cardiovascular system⁶¹. Previously, we have shown that ADAM10 is highly expressed in human atherosclerotic lesions and was seen to be associated with plaque progression and neovascularization⁶². Additionally, endothelial ADAM10 has been shown to take part in regulating endothelial permeability, leukocyte recruitment and angiogenesis⁶²⁻⁶⁵, processes that contribute in the progression of atherosclerosis. However, the role of endothelial ADAM10 in atherosclerosis development remains to be determined.

The role of ADAMs in inflammatory and angiogenic processes will be reviewed more extensively in chapter 3.

Upon shedding, the membrane protein remnant is subjected to further proteolytic processing in which an intracellular soluble fragment is generated after cleavage in its transmembrane domain (figure 3). This process is known as Regulated Intramembrane Proteolysis (RIPping). The resulting soluble intracellular domain (ICD) can be either directed to further degradation or can act as a signaling molecule in the cytosol or translocate to the nucleus where it can modulate transcriptional activity. RIPping is carried out by intramembrane-cleaving proteases (I-CLiPs), which, unlike other proteases, can cleave proteins within the hydrophobic environment of biological membranes. Currently, I-CLiPs are represented by four I-CLiP families, including the serine-, glutamyl-, metallo- and aspartyl-proteases^{52,66}.

One of the most intensively studied RIP events is the release of the Notch ICD, which is facilitated by the aspartyl protease presenilin, the catalytic component of the γ -secretase complex. In addition to Notch, presenilin can process numerous other membrane-bound type I-oriented proteins⁶⁷. In contrast, recently identified members of the aspartyl protease family, namely the signal peptide peptidase-like (SPPL) proteases, can exclusively cleave transmembrane proteins in the type II orientation and have a relatively unknown repertoire of substrates⁶⁸.

The SPPL family consists of four proteases, namely SPPL2a, SPPL2b, SPPL2c and SPPL3, each having distinct tissue expression and subcellular distribution patterns. To date, only SPPL2a and SPPL2b have been implicated in regulating inflammatory signaling⁶⁸. Tumour necrosis factor α was the first identified substrate relying on SPPL2a- and SPPL2b-dependent RIPping *in vitro*^{69,70}. Furthermore, CD74, which plays an important role in B lymphocyte development, antigen presentation as well as functioning as surface receptor for the cytokine macrophage migration inhibitory factor, was shown to be cleaved by both SPPL2a and SPPL2b *in vitro*^{55,71,72}. However, the processing of CD74 is dependent on SPPL2a *in vivo*, since genetic ablation of SPPL2a in mice cannot be compensated by SPPL2b⁵⁵.

Both shedding and RIPping are highly regulated processes, however their role in immune-mediated diseases, such as atherosclerosis, still remains to be defined.

Aim and outline of thesis

In this thesis, we aimed to investigate the immunomodulatory actions of HDL, and the effects of transmembrane proteolysis by ADAMs and SPPLs on vascular inflammation. HDL can exert a myriad of functions in many different cell types (figure 2). Though HDL dampens immune-related processes in endothelial cells, VSMCs and foamy macrophages, the immunomodulatory properties of HDL in non-lipid-laden macrophages have yet to be established, which will be investigated in **chapter 2 and 3**.

Post-translational proteolytic processing of transmembrane proteins by shedding and RIPping, resulting in the release of the extra- or intracellular domain, respectively, is emerging as an important mode of immunomodulation. Tight regulation of both processes is necessary to preserve homeostasis, however aberrant (de)activation may fuel pathological processes, including atherosclerosis development.

Chapter 4 gives an overview of the role of ADAM sheddases in cell migration and processes relying on cell migration, including immune cell recruitment, wound healing and cancer metastasis. In **chapter 5** we evaluated whether ADAM8, which is consistently upregulated in inflammatory diseases, including atherosclerosis, is causally involved in the pathogenesis in atherosclerosis.

Endothelial cells form a crucial barrier between components transported in blood and the underlying tissue. However, in inflammatory conditions, like atherosclerosis, endothelial cells become activated, leading to disruption of their barrier function and recruitment of immune cells. ADAM10 has previously been shown to be highly expressed in endothelial cells of atherosclerotic lesions and to play a role in endothelial permeability, leukocyte recruitment and angiogenesis. Therefore, in **chapter 6**, we investigated the role of endothelial ADAM10 in atherosclerosis development.

In contrast to ADAMs, the role of the recently identified intramembrane-cleaving aspartyl proteases, SPPL2a and SPPL2b, in health and disease, including atherosclerosis, remains ill-defined. Therefore, in **chapter 7**, we set out to uncover new endogenous substrates for SPPL2a and SPPL2b, and to determine whether they are involved in the development of atherosclerosis.

Finally, findings based on the experimental results from this thesis and their future implications will be discussed in **chapter 8**.

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Chapter 2

High-density lipoproteins exert pro-inflammatory effects on macrophages via passive cholesterol depletion and PKC-NF κ B/STAT1-IRF1 signaling

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Abstract

Membrane cholesterol modulates a variety of cell signaling pathways and functions. While cholesterol depletion by high-density lipoproteins (HDLs) has potent anti-inflammatory effects in various cell types, its effects on inflammatory responses in macrophages remain elusive. Here we show overt pro-inflammatory effects of HDL-mediated passive cholesterol depletion and lipid raft disruption in murine and human primary macrophages *in vitro*. These pro-inflammatory effects were confirmed *in vivo* in peritoneal macrophages from apoA-I transgenic mice, which have elevated HDL levels. In line with these findings, the innate immune responses required for clearance of *P. aeruginosa* bacterial infection in lung were compromised in mice with low HDL levels. Expression analysis, ChIP-PCR, and combinatorial pharmacological and genetic intervention studies unveiled that both native and reconstituted HDL enhance Toll-like-receptor-induced signaling by activating a PKC-NF- κ B/STAT1-IRF1 axis, leading to increased inflammatory cytokine expression. HDLs pro-inflammatory activity supports proper functioning of macrophage immune responses.

Introduction

Macrophages are crucial actors in innate immunity, employing pattern-recognition receptors such as Toll-like receptors (TLRs) which, upon ligand recognition, activate several signaling pathways regulating immune cell functions such as pro-inflammatory cytokine production, apoptosis, and phagocytosis ¹. Membrane cholesterol—more specifically, lipid rafts—plays a pivotal role in modulating signaling, including TLR pathways, and subsequent cell functions ². Indeed, cholesterol depletion by HDL is well known to have anti-inflammatory, anti-oxidant, and anti-apoptotic functions ³. Hence, plasma HDL levels have been inversely associated with clinical events resulting from atherosclerosis ⁴. We have previously shown that HDL reduces the expression of pro-inflammatory chemokines in endothelial cells (ECs) and smooth muscle cells (SMCs) ^{5,6}. Only a few studies have investigated the effects of HDL on inflammatory cells. Suzuki *et al.* ⁷ reported anti-inflammatory effects of native HDL (nHDL) on macrophage-derived foam cells, while others reported MyD88/p65-dependent pro-inflammatory effects of lipid-free apoA-I ⁸ or anti-inflammatory effects of apoA-I reconstituted with soybean phosphatidylcholine (PC) ⁹ on macrophages. Although activating transcription factor 3 (*Atf3*) has been implicated in HDL-mediated responses in the latter study, the signaling pathways affected by HDL in macrophage inflammatory responses largely remain to be elucidated.

Here we report that HDL augments TLR-induced, pro-inflammatory responses in murine and human macrophages, thereby enhancing the capacity to clear infection with *P. aeruginosa* bacteria in mice. We propose that HDL-induced cholesterol depletion and subsequent protein kinase C (PKC) activation lead to NF- κ B/STAT1-IRF1-dependent modulation of TNF- α , IL-12, and IL-10 production.

Results and Discussion

HDL enhances inflammation in primary macrophages

In keeping with literature^{5,10}, HDL significantly reduced TNF- α -elicited gene expression of chemotactic (*Ccl2* and *Ccl5*) and adhesion molecules (*Vcam-1* and *E-selectin*) (**Figure S1A**) in ECs, confirming HDL functionality. HDL functionality was further established in bone-marrow-derived macrophages (BMDMs) by the finding of 53% and 63% cholesterol efflux capacity of reconstituted HDL (rHDL) and nHDL, respectively, as shown previously¹¹. Surprisingly, rHDL treatment of BMDMs resulted in a significant, concentration-dependent increase in gene expression of pro-inflammatory *Il-12* and *Tnf- α* , whereas anti-inflammatory *Il-10* was significantly decreased (**Figure S1B**). Additionally, rHDL, nHDL, or commercially obtained HDL (cHDL) all had comparable, dose-dependent, pro-inflammatory effects in BMDMs at protein level (**Figures 1A, 1B, S1C, and S1D**). Interestingly, HDL's pro-inflammatory effects became evident after 6–12 hr HDL pre-incubation, ruling out any acute (i.e., cytotoxic or contamination) effects (**Figure 1C**). nHDL also enhanced secretion of IL-1 β , IL-5, IL-6, and nitric oxide (NO) by BMDMs (**Figure S1E**). *Vcam1*, *Ccl2*, *Ccl5*, or *Ifnb* mRNA and protein expression were not significantly altered by HDL (**Figure S1F**; data not shown). MTT assay analysis did not reveal any signs of cytotoxicity (data not shown), and all HDL preparations were negative for endotoxin (i.e., <0.01 EU [<1 pg] per 10 μ g apoA-I). Moreover, HDL treatment of non-stimulated BMDMs failed to induce inflammatory responses, in contrast with low-level LPS stimulation (**Figure S1G**). Finally, HDL treatment of BMDMs in the presence of the endotoxin-neutralizing agent polymyxin B had similar pro-inflammatory effects (**Figure S1H**). These data led us to exclude that endotoxin contamination of HDL preparations was responsible for HDL's pro-inflammatory activity. *In vitro* HDL treatment of resident mouse peritoneal macrophages (PMs) (**Figure S1I**) and human peripheral blood monocyte-derived macrophages (**Figure S1J**) resulted in comparable pro-inflammatory effects. In line with these findings, others have shown that nHDL was able to increase LPS-induced TNF- α mRNA expression in cholesterol-loaded PMs⁷.

Ex vivo HDL priming augments TLR-induced inflammation in macrophages

To corroborate whether these pro-inflammatory effects also manifest *in vivo*, we examined transgenic (Tg) mice with hepatic overexpression of human apoA-I, which results in increased HDL levels in plasma (data not shown) and peritoneal fluid (**Figures S2A and S2B**). Indeed, PMs isolated from LPS-treated apoA-I^{Tg} mice showed significantly increased pro-inflammatory TNF- α and IL-12 production compared to wild-type (WT) mice (**Figure 1D**). Interestingly, the HDL-associated, pro-inflammatory response was not restricted to TLR4 stimulation by LPS but was also observed for TLR1/2, TLR3, TLR7/8, and TLR9 (**Figures 1E and S2C**). HDL also enhanced *in vivo* TLR7/8 responses, since PMs isolated from R848 (TLR7/8 ligand)-treated apoA-I^{Tg} mice showed a similar pro-inflammatory trend (**Figure 1F**).

Figure 1. Pro-inflammatory functions of HDL on murine and human macrophages *in vitro* and *in vivo*

(A) Cytokine secretion by native HDL-treated and LPS-stimulated BMDMs is shown.
 (B) Cytokine secretion by 300 μg/ml native HDL-treated BMDMs using different LPS incubation times is shown.
 (C) Cytokine secretion by 300 μg/ml native HDL-treated and LPS-stimulated BMDMs using different HDL incubation times is shown.
 (D) *In vivo* HDL priming results in more inflammatory, LPS-triggered PMs re-stimulated *in vitro* with LPS ($n = 6$).
 (E) TNF-α cytokine secretion by HDL-treated WT BMDMs, stimulated with various TLR ligands, is shown.
 (F) *In vivo* HDL priming results in more inflammatory, R848-triggered PMs re-stimulated *in vitro* with R848 ($n = 6$).
 (G) *In vitro* *P. aeruginosa* phagocytosis by alveolar macrophages (MHS cell line) is shown. Representative image is of $n = 2$.
 (H) Bacterial titers in BALs are shown after *P. aeruginosa* infection over time.
 (I) HE-stained lung cross-sections after infection are shown.
 (J) BAL cell contents after *P. aeruginosa* infection are expressed as a ratio. Note the difference in the y axis, reflecting neutrophil influx over time. Statistical analysis uses Kruskal-Wallis with Dunn's post-test.
 (K) BAL fluid cytokine profile is shown after *P. aeruginosa* infection. Note the difference in the y axis. All results are expressed as mean $\pm$ SEM; n.d., not detectable. See also Figures S1 and S2.

A direct interaction of human lipid-free apoA-I with TLR2, TLR4, and MyD88 was previously shown to induce NF- κ B activation and pro-inflammatory TNF- α , IL-1, and IL-6 secretion in mouse PMs and human monocyte-derived macrophages⁸. Others, however, have reported anti-inflammatory effects of HDL, most likely attributable to different HDL sub-fractions that were shown to have contrasting effects on cells³. For instance, HDL₃ induced M2 macrophage markers and reduced *Il-6* and *iNos* expression¹², while soybean PC rHDL was reported to mediate *Atf3*-dependent, anti-inflammatory effects, at least at supra-physiological concentrations (~2-fold higher than average HDL levels in humans)⁹. We confirmed that soybean-derived PC rHDL reduced IL-12 and IL-10 production but that it did not affect TNF- α secretion, *Atf3*, or its target gene expression in BMDMs (at least within the 0–300 μ g/ml HDL range) (data not shown). As several soybean components are anti-inflammatory in macrophages¹³, the observed anti-inflammatory effects may reflect the soybean content of their rHDL preparation. De Nardo *et al.*⁹ also reported anti-inflammatory effects of nHDL treatment on plasma cytokine levels (IL-6 and IL-12) in C3H/HeJ mice, which carry a TLR4 mutation and display a defective response to bacterial endotoxins such as LPS. In our study, we show that both 1-palmitoyl-2-linoleoyl-PC-containing rHDL and nHDL exert pro-inflammatory effects in macrophages *in vitro* and *in vivo*, even at (sub-)physiological concentrations.

HDL modulates clearance of bacterial lung infection *in vivo*

To substantiate the biological relevance of HDL's pro-inflammatory effects on macrophage functions, we infected HDL-treated alveolar macrophages *in vitro* with *P. aeruginosa*, a bacterial strain of which elimination is predominantly dependent on proper macrophage functioning. HDL treatment significantly enhanced *P. Aeruginosa* phagocytosis by the macrophages (**Figure 1G**). After *in vivo* infection of WT, ApoA-I^{Tg}, or ApoA-I^{KO} mice, we observed that bacterial titers in the bronchoalveolar lavage (BAL) did not differ at 3 hr (**Figure 1H**) but were significantly lower at 24 hr in both WT and apoA-I^{Tg} mice (100 μ g/ml and ~400 μ g/ml HDL in lung, respectively, measured in BALs; **Figure S2D**) compared to apoA-I^{KO} mice (0.7 μ g/ml HDL) (**Figure 1H**); this suggests that low HDL levels lead to ineffective pro-inflammatory responses and bacterial clearance by macrophages. In line with this, lung inflammation was significantly increased (**Figure 1I**), while the macrophage:neutrophil ratio was significantly decreased (**Figure 1J**) at 24 hr after infection in apoA-I^{KO} mice compared to WT or apoA-I^{Tg} mice, concomitant with a delayed and defective innate immune response in mice with low HDL levels. Alternatively, HDL is known to exert anti-inflammatory effects on endothelium, i.e., reducing leukocyte adhesion molecule expression; this could also explain the reduced neutrophil recruitment in mice with high HDL levels (note that macrophages are not recruited within 24 hr). Moreover, increased neutrophil levels in apoA-I^{KO} mice might reflect impaired resolution of inflammation, which starts within the first hours of infection¹⁴. At 3 hr (when bacterial titers, **Figure 1H**, and BAL leukocyte content, **Figure S2E**, are similar), ApoA-I^{Tg} mice had a more pro-inflammatory BAL fluid cytokine profile (**Figure 1K**), i.e., a significant increase in IL-6 and mKC compared to apoA-I^{KO} mice; they also displayed a trend toward increased IL-1 β . At 24 hr after infection, however, ApoA-I^{KO} mice have increased levels of inflammatory cytokines in BAL fluid, most likely due to the persistence of high bacterial titers in these mice. IL-2 and IL-12 were below detection

limits.

Passive cholesterol efflux mediates pro-inflammatory HDL effects

HDL's pro-inflammatory activity in macrophages is not mediated by active cholesterol efflux, as deficiency of main mediators ABCA1, ABCG1, SR-B1, or CD36¹⁵ was unable to prevent the HDL-induced increase in LPS response (**Figure 2A**). However, compensation by non-ablated lipid transporters cannot be excluded.

HDL can also extract cholesterol from cells by passive diffusion, which has been shown to disrupt lipid rafts. HDL appeared to be as effective in extracting membrane cholesterol (40%–50%) as the cholesterol-depleting agent methyl- β -cyclodextrin (M β CD) (**Figure 2B**). Similarly, PMs from apoA-I^{Tg} mice had 40% less membrane cholesterol staining than WT PMs. Whether the lipid raft content in PMs of ApoA-I^{KO} mice is enhanced compared to WT mice remains to be determined. Interestingly, M β CD treatment of BMDMs showed effects similar to HDL on LPS-induced secretion of TNF- α and IL-10, although it did not affect IL-12 (**Figure 2C**); this suggests that the observed inflammatory response is at least partly associated with cholesterol extraction. Indeed, prior disruption of lipid rafts by pre-treatment with M β CD (but not by cholesterol-saturated M β CD pre-treatment) ablated the effects of HDL on TNF- α secretion, and HDL did not further reduce IL-10 secretion (**Figure 2C**).

Interestingly, lipid raft disruption (by M β CD as well as HDL) in ECs has been shown to enhance the activity of ADAM17, the major enzyme responsible for TNF- α release¹⁶. Although HDL slightly upregulated *Adam17* mRNA expression, it did not affect general ADAM activity (data not shown). TNF- α production, however, was completely blunted with combined ADAM10 and ADAM17 inhibition (GW280264X), but not with ADAM10 inhibition alone (GI254023X), confirming the ADAM17 dependence of TNF production in macrophages (**Figure 2D**). Whether lipid raft disruption by HDL in macrophages, like in ECs, leads to ADAM17 redistribution and thereby enhanced TNF release remains to be determined. Co-incubation with the soluble TNF receptor (Enbrel) to capture TNF- α did not impact HDL's IL-12 and IL-10 responses, refuting the idea that BMDM-released TNF- α is responsible for HDL's modulation of IL-10 and IL-12 secretion (**Figure 2E**).

Figure 2. Lipid Raft Disruption by Cholesterol Depletion Mediates Pro-inflammatory HDL Effects

(A) Cytokine secretion by HDL-treated and LPS-stimulated BMDMs is shown. The dotted line represents HDL's effect in WT BMDMs.

(B) Lipid raft staining of WT BMDMs or WT and Tg PMs and representative pictures of BMDMs are shown.

(C) Cytokine secretion by cholesterol depleted and LPS-stimulated BMDMs is shown. Additionally, BMDMs were pre-treated with M β CD or a M β CD and cholesterol mixture and washed before HDL incubation.

(D and E) Cytokine secretion by HDL-treated and LPS-stimulated BMDMs, with or without ADAM inhibition (D) or soluble TNFR Enbrel (E), is shown. All results are expressed as mean $\pm$ SEM.

Pro-inflammatory HDL effects are mediated by STAT1 and NF- κ B

Transcriptomic analyses revealed that while LPS, as expected, induced a range of pro-inflammatory genes and signaling pathways (data not shown), 51% of LPS-induced genes were further upregulated by nHDL (**Figure S3A**). M β CD largely, but not completely, mimicked HDL's effect (**Figure S3B**). HDL-regulated, LPS-induced genes revealed a strong enrichment of interferon regulatory factor 1 (IRF1) and NF- κ B-p65-dependent genes (**Figure 3A**), which were also found to be enriched among M β CD-regulated, LPS-induced genes (**Figure S3C**). Heatmaps of NF- κ B-responsive genes (**Figure 3B**), IRF1-responsive genes (**Figure 3C**), and immune pathway genes (**Figure S3D**) confirmed a general upregulation of LPS-induced genes by HDL, while these effects were less pronounced after M β CD incubation. As also illustrated by protein interaction analysis, signal transducer and activator of transcription 1 (STAT1) is a critical upstream regulator of IRF1 (**Figure S3E**). STAT1 deficiency significantly reduced the HDL-associated augmentation of IL-12 but did not reduce HDL-mediated modulation of IL-10 and TNF- α (**Figure 3D**), indicating that STAT1 is only partially contributing to HDL's pro-inflammatory effects.

Treatment of BMDMs with rHDL also resulted in significantly increased expression of NF- κ B target genes (**Figure S3F**). In addition, HDL treatment of BMDMs from NF- κ B luciferase reporter mice amplified the LPS-induced luciferase activity (**Figure 3E**) but did not affect baseline luciferase activity, confirming endotoxin-free HDL preparations (**Figure S3G**). While LPS stimulation induced phosphorylation (i.e., activation) of the NF- κ B sub-unit p65 (Ser536), this was significantly enhanced by M β CD, and the same trend was observed for HDL (**Figure S3H**). However, HDL did not enhance nuclear translocation of p65 (**Figure S3I**). Interestingly, p65 chromatin immunoprecipitation (ChIP) and subsequent quantitative PCR showed that p65 occupancy of the *Tnf- α* and *Il10* promoters was affected by HDL as well as M β CD, while that of *Il12* was not changed (**Figure 3F**), again indicative of divergent pathways for IL10 and TNF on the one hand and IL12 on the other. However, p65 occupancy of the *Il10* promoter was not affected by rHDL (**Figure 3E**). Therefore, rHDL-dependent regulation of NF- κ B responses potentially implicates another NF- κ B sub-unit. Furthermore, p65 binding to the *Nos2* promoter was increased by both HDL preparations, but not by M β CD.

Several upstream signaling pathways can affect NF- κ B activation¹⁷. HDL's effects on BMDMs required neither IKK α , as demonstrated in IKK $\alpha^{AA/AA}$ mice homozygously expressing a constitutively inactive variant of IKK α , nor NIK or CKII activity (**Figures S3J–S3L**). IKK β -LysMcre BMDMs showed no effects, which could be attributed to the only marginal reduction in IKK β expression (30.2% $\pm$ 5.2%). However, inhibition of IKK β (and IKK α) partially quenched the effects of HDL on IL-10 secretion, but not on

TNF- α and IL-12 secretion (**Figure 3G**). IKK inhibition exacerbated the HDL effect on TNF- α , though absolute cytokine secretion was reduced due to IKK inhibition (data not shown). These data suggest that HDL enhances the LPS-induced NF- κ B transcriptional activity but not its nuclear translocation, at least partly dependent on IKK β .

HDL's pro-inflammatory effects depend on PKC activity

A well-known lipid-raft-associated kinase implicated in direct NF- κ B activation and STAT1 phosphorylation is PKC¹⁸. While several PKCs (like PKC δ and PKC ζ) have been shown to act through IKK, direct activation of NF- κ B has also been reported for some PKC isoforms¹⁹. nHDL increased PKC activity in BMDMs (**Figure 4A**), a phenomenon also observed with M β CD (data not shown). Co-incubation of BMDMs with HDL and Ro31-8425, a broad spectrum PKC inhibitor, completely blunted HDL-associated, pro-inflammatory effects (**Figure 4B**). BMDMs express all the conventional PKC isoforms, PKC α , PKC β , and PKC γ ; the novel PKCs, PKC δ , PKC ϵ , and PKC η (while PKC θ was undetectable); and the atypical PKCs, PKC ι and PKC ζ (**Figure S4**). In line with the previously reported redundancy for PKC isoforms^{20,21}, we demonstrated—using a range of pharmacological inhibitors and genetic knockout models—that HDL's pro-inflammatory effects depend on a combination of PKC isoforms rather than on single PKC isoforms (**Table S1**). Indeed, inhibition of all conventional PKCs (PKC α , PKC β , and PKC γ) appears to be required to block HDL's effect on IL-12 (**Figure 4C**), as is evident at low concentrations of UCN-01 (IC₅₀s for these PKCs is $\sim$ 30 nM), while inhibition of only PKC α and PKC β by Gö6976 is not sufficient (**Figure 4D**). At higher concentrations, UCN-01 inhibits not only the conventional but also the major novel PKCs, PKC δ and PKC ϵ (IC₅₀s $\sim$ 550 nM), which apparently have an opposite effect on HDL's induction of IL-12 than do PKC α , PKC β , and PKC γ . Interestingly, while lower concentrations of UCN-01 as well as PKC α and PKC β inhibition by Gö6976 were ineffective, higher concentrations of UCN-01 did block HDL's effects on IL-10 and TNF (**Figures 4C and 4D**), confirming that a combination of conventional and novel PKC isoforms is required for HDL's effects on these cytokines. In line with this, neither Gö6976 nor a pseudo-substrate inhibitor of PKC δ , PKC ϵ , and PKC θ (**Figure 4E**) affected HDL's effect on IL-10, whereas the combination of both did (**Figure 4F**). Similar findings were observed for TNF, although the pseudo-substrate for PKC δ , PKC ϵ , and PKC θ alone could already partially block HDL's effect on TNF (**Figure 4E**).

In conclusion, HDL's pro-inflammatory effects in macrophages are mediated by the concerted activity of multiple PKC isoforms. While the conventional PKCs—PKC α , PKC β , and PKC γ —seem mainly responsible for HDL's effect on IL-12, the combination of both conventional and novel PKCs seems to be required for it to affect TNF and IL-10. It remains to be determined whether the involved PKCs act through IKK or directly phosphorylate NF- κ B. The latter has been demonstrated for PKC ϵ and leads to enhanced p65 transcriptional activity, most likely by influencing recruitment of transcriptional (co-)regulators rather than influencing p65 translocation itself²², which concurs with our p65 findings. Interestingly, PKC was also shown to directly phosphorylate and thus activate STAT1¹⁸, which we identified as an additional transcriptional regulator at least partially involved in HDL's effects on IL-12 secretion.

Figure 4. PKC Mediates HDL's Pro-inflammatory Effects in Macrophages

(A) PKC activity in BMDMs incubated with nHDL or Ro31-8425 is shown.

(B–F) Cytokine secretion by HDL-treated and LPS-stimulated BMDMs is shown with or without Ro31-8425 (B), UCN-01 (C), Gö6976 (D), PKC δ , PKC ϵ , and PKC θ inhibitor (E), or both Gö6976 and PKC δ , PKC ϵ , and PKC θ inhibitor (F).

(G) Proposed (simplified) mechanism of pro-inflammatory action of HDL in macrophages is shown.

All results are expressed as mean $\pm$ SEM. See also Figure S4 and Table S1.

Collectively, the data led us to propose the following, simplified mode of action of HDL's immunomodulatory effects in macrophages (**Figure 4G**). HDL extracts membrane cholesterol independent of known cholesterol transporters or scavenger receptors, thereby disrupting lipid rafts. This, in turn, will activate multiple PKC isoforms, leading to increased production of pro-inflammatory mediators such as IL-12 and TNF- α and a reduction in IL-10. These overall pro-inflammatory effects require NF- κ B/p65 (TNF- α and IL-10) and are at least partly dependent on STAT1/IRF1 signaling (IL-12). Although additional pathways and transcriptional (co-)regulators may be implicated as well, our studies point to a concerted action of multiple conventional and novel PKCs to mediate HDL's effects on TNF- α and IL-10.

HDL-amplified, pro-inflammatory responses appear to be specific for macrophages, since HDL exerts anti-inflammatory effects in other vascular cell types^{5,6}. It is conceivable that HDL levels can impact various disorders involving macrophage responses, as was also indicated both by our *in vivo* bacterial infection with *P. aeruginosa* and by a study showing increased susceptibility of apoA-I^{KO} mice to septic death²³. Thus, our study legitimizes a thorough assessment of HDL-associated effects on macrophage inflammatory responses and clinical outcomes. The relevance of our findings for atherosclerosis and heart disease is difficult to assess. Though a more pro-inflammatory macrophage phenotype is generally considered to be detrimental for plaque progression and stability, it may well have beneficial effects in promoting clearance of plaque lipids and debris. Moreover, the overt anti-inflammatory effects in other cell types should be taken into account. It is the balance between these opposite effects of HDL that will determine clinical outcome for cardiovascular disease patients. Nevertheless, the disappointing outcomes from recent clinical trials and a meta-analysis integrating data from 39 clinical trials on the efficacy of HDL-targeting therapy²⁴ at least raise a note of caution, and underline the importance of a better understanding of HDL's full activity spectrum in complex multifactorial diseases.

Materials and Methods

See supplemental material for more details.

Preparation of native HDLs and discoidal reconstituted HDLs. Spherical, native HDLs were commercially bought (cHDL, GenWay Biotech) or isolated from human plasma by ultracentrifugation (nHDL). Discoidal rHDL containing apoA-I complexed to 1-palmitoyl-2-linoleoyl-PC (Avanti Polar Lipids), initial phospholipid/apoA-I molar ratio 100:1, were prepared by the cholate dialysis method. HDL concentrations are given as total apoA-I protein..

Animals. All animal experiments were approved by the Animal Ethics Committee of the Maastricht University (permit numbers 2010-178/2012-090). All mice were 9-16 weeks old and on a C57BL/6 background.

Cell culture and treatments. Human Coronary Artery ECs (Lonza) were cultured until 90% confluency. Bone marrow was differentiated into BMDMs using 20% (vol/vol) L929-conditioned medium for 8-9 days. Resident PMs were obtained by flushing the peritoneal cavity with ice-cold PBS. Human monocyte derived macrophages were isolated from healthy donor buffy coats.

Cells were incubated with 300µg/ml HDL for 24h or 4% (vol/vol) MβCD for 4h, washed and stimulated with 10ng/ml LPS for 6 (TNF-α, IL-10, CCL2, IFNβ) or 24h (IL-12) for ELISA analysis, unless stated otherwise.

Gene expression. RNA was isolated using the High Pure RNA Isolation Kit (Roche), according to manufacturer's instructions and reverse transcribed using iScript (BioRad). Quantitative PCR was performed using SensiMix (Quantace-Bioline). Cyclophilin A and β-actin were used as housekeeping genes. Primer sequences available on request.

Enzyme-Linked Immunosorbent Assay (ELISA). IL-12(p40), TNF-α, CCL2 and IFNβ (Invitrogen), IL-10 (eBioscience) and apoA-I (Mabtech and USCN Life Science) ELISA assays were performed on conditioned medium according to manufacturer's instructions.

NO assay. NO production was measured in conditioned medium using Griess reagent (2.5% (vol/vol) H₃PO₄, 1% (wt/vol) sulfanilamide, 0.1% (wt/vol) naphthalene diamine dihydrochloride). Analysis was performed using a micro-plate reader (Bio-Rad) at 550 nm.

Cholesterol efflux assay. BMDMs were incubated for 24h with ³H-cholesterol. Cells were washed and equilibrated for 1h, followed by 24h 0.2% (wt/vol) BSA or 300ug/ml HDL incubation, after which radioactivity was measured in the media and cell lysates. Cholesterol efflux is expressed as the percentage of total cell ³H-cholesterol present in the medium.

Flow cytometry analysis. WT and apoA-I^{Tg} mice were intra-peritoneally stimulated with 20µg LPS (48h) or 10µg R848 (2h) prior to isolation of resident PMs. After overnight attachment, floating cells were removed while attached cells were activated for 9h by 10ng/ml LPS or 10µg/ml R848 in combination with GolgiSTOP (Beckton Dickinson). Flow cytometry analysis was subsequently performed according to described protocol²⁵.

In vitro phagocytosis. Mouse alveolar macrophage cell line MHS (CRL-2019; ATCC) was treated with 0-300µg/ml HDL for 24h, washed and infected with WT *Pseudomonas aeruginosa* strain K (PAK strain, MOI=10) for 1h. Free and adherent bacteria were removed by washing cells with PBS, the cells were lysed in 0.1% Triton X-100 and the number of bacteria in lysates was determined by counting colony-forming units (CFU) on LB agar plate.

Bacterial infection model. WT, apoA-I^{Tg} and apoA-I^{KO} mice were intra-tracheally infected with the WT *Pseudomonas aeruginosa* strain K (PAK strain, 10⁶ CFU/mouse) and 3h (n=6) or 24h (n=5-7) later BAL were performed as described²⁶. Bacterial colonies and differential cell counts were checked by streaking serial diluted BALs on LB-agar plate and using cytospin preps with Giemsa stains, respectively. Lungs were dissected, fixed in 1% paraformaldehyde and embedded in paraffin. Serial sections (4 µm) of lung tissue were cut and stained with haematoxyline-eosine (HE) for qualitative examination of amount of inflammatory cells (using scores from 0 (absent) to 3 (high abundance)).

Cholera toxin staining. For lipid raft staining, BMDMs from WT mice or PMs from WT and apoA-I^{Tg} mice were plated on Lab-Tek borosilicate 8-chambered coverglasses (Thermo Fisher Scientific, Nunc) at a density of 2x10⁵ cells/well. Staining was performed using the Vybrant® Lipid Raft Labeling kit (Life Technologies, Molecular Probes) according to the manufacturer's protocol. Pictures were taken using an Axiovert LSM 510 or Zeiss LSM Live 7 confocal microscope (Zeiss) at a 63x magnification.

Genome-wide microarray analysis and bioinformatics. RNA was isolated, processed and hybridized with the Mouse WG-6 v2.0 Beadchips (Illumina) at Aros Applied Biotechnology (GSE70155, Aarhus, Denmark). Analysis was performed using MATLAB, HOMER and Topp-Fun.

NFκB Luciferase assay. Assay was performed using BMDMs from NFκB Luciferase reporter mice, according to the manufacturer's protocol (Promega), and measured using a GloMax-96 Luminometer (Turner Biosystems).

Western blotting. Cells were lysed and homogenized in lysis buffer (1% (vol/vol) Triton, protease inhibitor, PhosSTOP, 150mM NaCl, 200mM Tris and glycerol). Equal amounts of protein were separated on an 8% (vol/vol) SDS-PAGE gel by electrophoresis and transferred to a nitrocellulose membrane. Membranes were probed with described antibodies (suppl. material). Chemiluminescence was detected using a digital scanner.

P65 Chromatin Immunoprecipitation (ChIP). p65 ChIP was performed on 40×10^6 cells per condition, as described previously ²⁷. ChIP-qPCR was performed using Sybr Green Fast (Applied Biosystems) on a ViiA7 PCR machine (Applied Biosystems). Relative enrichments are presented as percentage input. Primer sequences available on request.

PKC activity assay. PKC activity assay (Enzo Life Sciences) was performed on cell lysates according to manufacturer's instructions.

Statistical analysis. Data are presented as mean $\pm$ standard error of the mean (SEM). All statistical analyses were performed using Prism (GraphPad Software Inc). All data are from three independent experiments unless stated otherwise. Statistically significant differences were evaluated with the Student's t-test unless stated otherwise. Significance was accepted at the level of $P < 0.05$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ denote significant differences compared to non-HDL pre-incubated control cells. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ denote significant differences compared to WT cells.

Accession numbers

The accession number for the data reported in this paper is GEO: GSE70155.

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Supplemental figures

Figure S1. Endotoxin-free HDL has anti-inflammatory effects in HCAECs, though pro-inflammatory effects on mouse and human macrophages *in vitro* and *in vivo*.

(A) mRNA expression in HCAECs pre-treated with nHDL (600µg/ml) for 24h and stimulated with TNF-α (2.5ng/ml) for 5h quantified by PCR (representative figure of $n = 3$)

(B) mRNA expression in BMDMs incubated with HDL

(C) Cytokine secretion by rHDL-treated/LPS stimulated BMDMs

(D) Cytokine secretion by commercial HDL-treated/LPS stimulated BMDMs

(E) Multiplex immunoassay of cytokine secretion and Griess assay of NO secretion by HDL-treated/LPS stimulated BMDMs

Figure S1 continued. Endotoxin-free HDL has anti-inflammatory effects in HCAECs, though pro-inflammatory effects on mouse and human macrophages *in vitro* and *in vivo*.

(F) Cytokine secretion by HDL-treated/LPS stimulated BMDMs

(G) Cytokine secretion by HDL-treated/LPS, unstimulated or LPS (1ng/ml) stimulated BMDMs ($n = 2$)

(H) Cytokine secretion by HDL-treated/LPS stimulated BMDMs in the absence or presence of polymyxin B

(I-J) Cytokine secretion by HDL-treated/LPS stimulated resident PMs (E) or human monocyte derived macrophages (J). All results are expressed as mean $\pm$ SEM

Figure S2. ApoA-I levels in mice and HDL modulated cytokine secretion after TLR activation

(A) ApoA-I level in peritoneal fluid isolated from wildtype and apoA-I transgenic mice ($n = 6$) after LPS trigger

(B) ApoA-I level in peritoneal fluid isolated from wildtype and apoA-I transgenic mice ($n = 6$) after R848 trigger

(C) Cytokine secretion by HDL-treated BMDMs, stimulated with various TLR ligands

(D) ApoA-I level in BAL fluid isolated from wildtype, apoA-I knockout and apoA-I transgenic mice 24h after *P. aeruginosa* infection

(E) BAL neutrophil and macrophage contents 3h after *P. aeruginosa* infection expressed as ratio.

All results are expressed as mean $\pm$ SEM

Figure S3. NF- κ B and interferon signaling pathways are crucial mediators in the macrophage pro-inflammatory HDL effects.

(A-B) LPS-induced genes differentially regulated by HDL or M β CD (A) and overlap in these genes (B) identified by microarray analysis in HDL treated, 6h LPS stimulated BMDMs

(C) *De novo* motif analysis of LPS-induced genes further upregulated by methyl- β -cyclodextrin.

(D) Microarray expression levels of aberrant immunity phenotype represented as heat maps (red/green indicates increased/decreased expression, respectively, compared to average of 3 LPS conditions)

(E) DAVID STRING protein-protein interaction network created by entering IL-1b, IL-6, IL-10, IL-12, TNF- α , Nos2 and the identified IRF1 and p65/Rela transcription factors as proteins of interest, while automatically selecting 10 additional interacting partners. Different line colors represent the types of evidence for the association

(F) mRNA expression in BMDMs incubated with HDL

(G) Luciferase levels in BMDMs from NF- κ B luciferase reporter mice stimulated with 0-0.1ng/ml LPS for 3h or incubated with HDL only for 24h

Figure S3. continued

(H) Immunoblot showing phosphorylation of NF-κB subunit p65 (p-p65) after HDL or MβCD treatment followed by 5min stimulation with 10ng/ml LPS. Representative immunoblot is shown with quantification of *n* = 5 experiments
(I) Immunofluorescent images of NF-κB subunit p65 after HDL treatment followed by 1h stimulation with 10ng/ml LPS. Representative images of *n* = 3 are shown. Quantification of p65 translocation, measured by cytosolic/nuclear p65 ratio is given
(J-L) Cytokine secretion by HDL-treated/LPS stimulated IKK^{AAA/AA} (J), NIK^{-/-} (K), CKII inhibited (L) BMDMs
All results are expressed as mean ± SEM

Figure S4. PKC isoform expression in BMDMs.
Gene expression in BMDMs after 6h LPS stimulation

Table S1. Overview of the combinatorial approach used to investigate PKC isoforms.

An overview is given of the various genetic knockout models, and a range of pharmacological inhibitors used to investigate specific PKC isoforms. Fold changes of HDL treatment compared to PBS control are showed. Dark Red indicates a significantly increased cytokine secretion upon HDL treatment, whereas green indicates a significantly reduced IL-10 secretion upon HDL treatment. No color indicates that the HDL effect is abrogated. Light Red indicates that the TNF levels of HDL-inhibitor treated cells significantly differ from the HDL-DMSO treated cells.
* Denotes *P* < 0.05, (*)Denotes *P* = 0.07

	Inhibitors / Knockout mice	PKC isoforms									TNF-α	IL-12	IL-10
		Conv.			Novel			Atyp					
		α	β	γ	δ	ε	η	θ	ζ	ι			
No Inh.	-										1.80***	2.37**	0.55***
Broad Spec.	Ro31-8425										1.02	1.06	0.85
Individual	PKCγ ^{-/-} mouse										1.84***	2.44***	0.67***
	PKCδ ^{-/-} mouse										1.77**	2.59**	0.52**
	PKCε ^{-/-} mouse										1.73(*)	3.32**	0.72**
	PKCη inhibitor										1.85**	2.89***	0.60*
Combinatorial	PKCι/ζ inhibitor										2.04***	2.31**	0.62*
	Gö6976										2.61***	2.29***	0.64***
	PKCδ/ε/θ inhibitor										1.43**	1.81**	0.48***
	Gö6976 + PKCδ/ε/θ inhibitor										1.37*	1.75**	1.22
	UCN-01 (0.1μM)										1.42**	1.19	0.80**
	UCN-01 (1μM)										1.13	3.05***	1.17

Supplemental information

Reagents. 1mg/ml Cholesterol / 4% (vol/vol) M β CD mixture was prepared by vortexing at 37°C (50 mg/ml stock solution of cholesterol in ethanol).

Table: Overview of used reagents and inhibitors

Reagent / inhibitor	Company	Concentration
β -actin antibody	Cell Signaling	1:1,000
Casein kinase II-inhibitor	Merck Millipore	10 μ g/ml
Cholesterol	Sigma	1mg/ml
CpG	Invivogen	10 μ M
Enbrel	Amgen	10 μ g/ml
GI54023X	Glaxo Smith Kline	5 μ M
Gö6976	Calbiochem	10 μ M
GW280264X	Glaxo Smith Kline	5 μ M
IKKVII	Calbiochem	2 μ M
Methyl- β -cyclodextrin	Sigma	4% (vol/vol)
Pam ₃ CSK ₄ (PAM)	Invivogen	10 μ M
phospho-NF- κ B p65 antibody	Cell Signaling	1:1,000
PKC- $\delta/\epsilon/\theta$ inhibitor	Calbiochem	10 μ M
PKC- η pseudosubstrate inhibitor	Calbiochem	10 μ M
PKC- ζ pseudosubstrate inhibitor	Santa Cruz	10 μ M
Poly:IC	Invivogen	2.5 μ M
Polymyxin	Life Technologies	10 μ g/mL
R848	Invivogen	10 μ M
rabbit IgG-HRP antibody	Cell Signaling	1:1,000
Ro31-8425	Merck Millipore	10 μ M
Rottlerin	Calbiochem	1 μ M
UCN-01	Sigma	0.01-1 μ M
Ultrapure LPS (E.coli 055:B5)	Sigma	1-10ng/ml

Preparation of discoidal reconstituted HDLs. To prepare discoidal reconstituted HDLs (rHDLs) human plasma was ultracentrifuged, delipidated and subjected to anion-exchange chromatography using a Fast Protein Liquid Chromatography (FPLC) system to isolate apoA-I ¹. Discoidal rHDL containing apoA-I complexed to 1-palmitoyl-2-linoleoyl-phosphatidylcholine (PLPC, Avanti Polar Lipids), initial phospholipid/apoA-I molar ratio 100:1, were prepared by the cholate dialysis method ². The protein concentration of apoA-I was determined using the nanodrop (Thermo Scientific). Phospholipid concentrations were enzymatically determined (Wako) ³. Limulus test was performed to exclude potential endotoxin contaminations (HyCult biotechnology).

Animals.**Table:** Overview of used mouse strains

Mouse strain	Generously donated by/purchase from
C57BL/6 (WT)	Charles River Laboratories
Human apoA-I ^{-/-}	Jackson Laboratories
Human apoA-I transgenic mice ⁴	Jackson Laboratories
ABCA1 ^{-/-}	Dr. Van Eck
ABCG1 ^{-/-}	Dr. Van Eck
SR-BI ^{-/-}	Dr. Van Eck
CD36 ^{-/-}	Dr. Koonen
IKK ^{AA/AA}	Dr. Lawrence
NIK ^{-/-}	Dr. Tas
STAT1 ^{-/-}	Dr. Mathieu and Dr. Gysemans, Leuven University, Leuven, Belgium
PKCδ ^{-/-}	Dr. Leitges
PKCε ^{-/-}	Dr. Parker, Francis Crick Institute, UK
PKCγ ^{-/-}	Dr. Regehr, Harvard Medical School, Boston, USA
NF-κB Luciferase mice	Dr. De Winther

Cell culture and treatments. Human Coronary Artery Endothelial Cells (HCAECs; Lonza) were cultured using EGM-2 MV BulletKit (Lonza) and grown until 90% confluent. Cells were incubated with HDLs for 24h, washed and stimulated with 2.5ng/ml TNF-α (R&D systems) for 5h.

Bone marrow was isolated from femurs and tibiae of described mice. Cells were cultured in RPMI-1640 medium (GIBCO Invitrogen) supplemented with 10% (vol/vol) heat inactivated fetal calf serum (Bodinco B.V.), 100U/ml penicillin, 100μg/ml streptomycin, 2mM L-glutamine (All GIBCO Invitrogen) and 20% (vol/vol) L929-conditioned medium (LCM) for 8-9 days to differentiate into bone marrow-derived macrophages (BMDMs). Macrophages were incubated with various concentrations of HDLs for 24h, washed and stimulated for 6 (TNF-α, IL-10, CCL2 or IFNβ) or 24h (IL-12) with 0-10ng/ml LPS.

Resident peritoneal macrophages were obtained by flushing the peritoneal cavity with ice-cold PBS followed by culturing in RPMI 1640 culture medium containing 10% (vol/vol) FCS, penicillin (100 U/ml), streptomycin (100ug/ml), and L-glutamine 2mM (all GIBCO Invitrogen). After overnight attachment, floating cells were removed while attached cells were incubated with various concentrations of HDLs for 24h, washed and stimulated for 6 (TNF-α or IL-10) or 24h (IL-12) with 0-10ng/ml LPS.

Human monocyte derived macrophages were obtained by isolating mononuclear cells from healthy donor buffy coats (Sanquin) using Lymphoprep (AXIS-SHIELD) density gradient centrifugation. Monocytes were further isolated by Standard Isotone Percoll gradient centrifugation (Amersham) and plated in Iscove modified Dulbecco medium

(IMDM, Life Tech), supplemented with 1% (vol/vol) fetal bovine serum (FBS, Life Tech) for 30min at 37°C. Non-adherent cells were removed, and monocytes cultured for 7 days in IMDM containing 10% (vol/vol) FBS, 1% (vol/vol) penicillin/streptomycin (Life Tech) and 10ng/ml M-CSF (Miltenyi). Macrophages were incubated with various concentrations of HDLs for 24h, washed and stimulated for 6 (TNF- α or IL-10) or 24h (IL-12) with 0-10ng/ml LPS.

Multiplex Immunoassay. IL-1 β , IL-2, IL-5, IL-6, IL-10, IL-12p70, IFN γ , and mKC multiplex ELISA (mouse pro-inflammatory panel1 V-PLEX™ Kit were performed on conditioned medium, mouse plasma or BAL fluid samples and measured using the SECTOR Imager 2400 according to manufacturer's instructions. (all Mesoscale Discovery).

Cholera toxin staining. Cells were washed with cold, serum-free DMEM and incubated with Alexa488-conjugated cholera toxin subunit B (CT-B) for 10min at 4°C, followed by lipid raft crosslinking with an anti-CT-B antibody for 15min at 4°C. Subsequently, the cells were fixed with 4% (vol/vol) Antigenfix (DiaPath) for 10min on ice and nuclei were stained with TO-PRO-3. Total cell fluorescence was measured for each cell within six fields of view per treatment using ImageJ software and corrected for background fluorescence (corrected total cell fluorescence = total cell fluorescence – (average background fluorescence x total cell area)).

Genome-wide microarray analysis and bioinformatics. Statistical Analysis was performed by means of MATLAB Statistical Toolbox. Afterwards, computed p-values were corrected using the Benjamini-Hochberg correction (FDR-Correction). MATLAB and its set operations functionality were used in order to analyze the intersection between differentially expressed genes when comparing LPS-induced environment to the effects of adding either HDL or Cyclodextrin. Intersection and set differentiation functions generated the numbers that were plotted on the Venn diagrams. Motif analysis was performed with HOMER⁵ using default settings. Heatmaps were generated using the R programming environment and RColorBrewer and gplots packages. Expression values were adjusted based on the average expression level of the gene in LPS alone condition. Protein-protein interaction network of the identified IRF1 and p65/Rela transcription factors was created using DAVID STRING (<http://www.string-db.org>).

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Chapter 3

Disease- or storage-associated structural modifications are unlikely to explain HDL pro-inflammatory effects on macrophages

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Main Text

High-density lipoproteins (HDLs) have been attributed to overt anti-inflammatory functions in a range of cell types, including endothelial cells ¹ and smooth muscle cells ². In our recent publication, we have shown that HDLs exert pro-inflammatory actions on macrophages, both *in vitro* and *in vivo*, and unraveled the underlying mechanisms and signaling pathways ³. These findings swirled up discussions about HDL, particularly as these findings, although concordant with other reports ⁴, seemed at odds with studies showing largely anti-inflammatory effects of HDL on innate immune cells.

In their letter, Kopecky *et al.* ⁵ stress the importance of HDL origin, handling, and storage conditions. We fully acknowledge the importance of these aspects, especially when studying immune responses. Indeed, HDL comprises a heterogeneous population, as its lipid, (micro)RNA, and protein composition highly depend on (health) conditions of the HDL donor, the method of isolation or preparation, and, as shown by Kopecky *et al.*, storage conditions. As rightly stated by Kopecky *et al.*, HDL covalent modifications (e.g., carbamylation, oxidation, etc.) or significant changes in protein and/or lipid composition, including the incorporation of serum amyloid A (SAA), commonly occur in diseases such as atherosclerosis or chronic kidney disease. Such modifications have been reported to render HDL dysfunctional ⁶, i.e., defective cholesterol efflux capacity or loss of anti-inflammatory capacity, as Kopecky *et al.* indeed demonstrated. As suggested by Kopecky *et al.*, the LPS challenge we used in our *in vivo* experiment and subsequent acute phase response may indeed have led to SAA accumulation in HDL; however, we could also observe pro-inflammatory effects of *in vivo* HDL priming on macrophages from apoA-I transgenic mice without *in vivo* LPS challenge (**figure S1**).

While we cannot fully rule out the aforementioned structural modifications of the HDL preparations, our study provides three lines of evidence to exclude the idea that these factors are responsible for the observed pro-inflammatory effects. First, we used several batches of native HDL (nHDL) (eight different isolations of a pool of ~15–20 healthy individuals, as well as commercially available (cHDL) and reconstituted HDL (rHDL). The age and health status of these volunteers render it highly unlikely that all different donors and batches (and donors used for apoA-I isolation/rHDL preparation) were enriched in SAA, thereby influencing our results. Second, oxidation, SAA incorporation, or other structural changes (due to storage or donor condition) would render HDL dysfunctional ⁶. However, cholesterol efflux was within normal (published) ranges (63% for nHDL; 53% for rHDL; ~40% reduced lipid raft staining in macrophages from apoA-I Tg mice), indicating that our HDL preparations are still functional. Third, and most importantly, Kopecky *et al.* convincingly showed that older HDL preparations lost their anti-inflammatory effects (though they did not induce pro-inflammatory effects), while our nHDL and rHDL preparations still showed anti-inflammatory effects in endothelial cells.

Rather than the above-mentioned structural modifications, we have good reason to believe that the discrepancies between the reported pro- and anti-inflammatory effects of HDL in macrophages are related to methodological differences of these studies. Roughly,

they can be classified into three main categories:

- (1) **Macrophage model:** Suzuki *et al.* ⁷ reported inhibitory effects of HDL (mainly at gene expression level) on the type I interferon response in acetylated LDL-loaded foam cells, while we ³, Smoak *et al.* ⁴, and De Nardo *et al.* ⁸ used non-lipid-loaded peritoneal, bone marrow-derived, and human monocyte-derived macrophages. While our study and that by Smoak *et al.* observed pro-inflammatory effects, De Nardo *et al.* reported anti-inflammatory effects, though with a different HDL preparation and concentration.
- (2) **HDL preparations and concentrations:** We used native HDL and 1-palmitoyl-2-linoleoyl-phosphatidylcholine reconstituted HDL (100–300 µg/mL), similar to Suzuki *et al.*, while Smoak *et al.* used lipid-free apoA1. De Nardo *et al.* showed anti-inflammatory effects of a soybean-phospholipid reconstituted HDL compound (CSL-111), which was attributed to *Atf3* upregulation, albeit at much higher HDL concentrations than used in our study (up to 2,000 µg/mL). We confirmed that rHDL composed of soybean-derived phospholipids reduced IL-12 and IL-10 production, without affecting TNF-α secretion and *Atf3* expression in macrophages (0–300 µg/mL HDL range) ³. As it has already been shown by Kang *et al.* ⁹ that several soybean components have anti-inflammatory activities in macrophages, the observed anti-inflammatory effects observed by De Nardo *et al.* may be derived from soybean constituents of their rHDL preparation.
- (3) **Readout model:** De Nardo *et al.* did also use native HDL, showing anti-inflammatory effects in vivo, though these data were based on whole plasma cytokine analysis (IL-6 and IL-12) in TLR4 mutant C3H/HeJ mice, which display a defective response to bacterial endotoxins like LPS (as used in our study), in contrast to the C57BL/6 mice used by Smoak *et al.*, Suzuki *et al.*, and our study.

Based on these substantial differences in methodology, it is rather challenging, if not nearly impossible, to make a straightforward comparison between the different studies. To fully elucidate the reasons behind the observed discrepancies, additional studies directly comparing different methodological approaches are necessary.

In our view, the relevance of these methodological differences is highlighted once again in the experimental data shown by Kopecky *et al.* As compared to fresh samples, older HDL preparations have an impaired ability to inhibit inflammatory cytokine secretion and co-stimulatory molecule expression in human monocytes and dendritic cells, respectively. In their study, the authors have investigated rather acute effects of HDL, as pre-incubation of only 90 min was used. In contrast, as we showed, pro-inflammatory effects of HDL in macrophages will not manifest within 90 min of pre-incubation; rather, they will become evident after 6–12 hr of pre-incubation, probably reflecting the time needed for sufficient cholesterol depletion. This confirms that minor differences in experimental setup may have deep impact on the outcome.

Taken together, although Kopecky *et al.* raise some important notes of caution regarding HDL source and processing and underpin the relevance of HDL quality control, the above-mentioned arguments conclusively rebut the notion that disease- or storage-associated structural modifications that render HDL dysfunctional explain the observed pro-inflammatory effects on macrophages.

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Supplemental figure

Figure S1. Pro-inflammatory effects of HDL after in vivo HDL priming without in vivo LPS challenge. Bone marrow derived macrophages, isolated from unchallenged wildtype (C57BL/6) and apoA-I transgenic mice were *in vitro* stimulated with LPS and cytokine expression was analyzed using FACS. Data are presented as mean $\pm$ standard error of the mean (SEM). Statistical significance of differences was evaluated with the Student's *t*-test.. **P* < 0.05, compared to WT cells.

Supplemental information

Animals. All animal experiments were approved by the Animal Ethics Committee of the Maastricht University (permit numbers 2010-178/2012-090). All mice (wildtype and human ApoA-I transgenic mice, both Jackson Laboratories) were 9-16 weeks old, mixed gender, on a C57BL/6 background and randomly assigned to experimental groups.

Bone marrow derived macrophage culture. Bone marrow was isolated from femurs and tibiae of described mice. Cells were cultured in RPMI-1640 medium (GIBCO Invitrogen) supplemented with 10% (vol/vol) heat inactivated fetal calf serum (GIBCO Invitrogen), 100U/ml penicillin, 100 μ g/ml streptomycin, 2mM L-glutamine (All GIBCO Invitrogen) and 20% (vol/vol) L929-conditioned medium (LCM) for 8-9 days to differentiate into bone marrow- derived macrophages (BMDMs).

Flow cytometry. Macrophages were incubated for 9h by 10 ng/ml ultrapure lipopolysaccharide (Sigma, E.coli 055:B5) *in vitro* in combination with GolgiSTOP (1:2000, BD Biosciences). Cytokine production was determined in macrophages as identified in the F4/80+ cell population (AbD, clone: CI:A31) by measuring IL-10 (eBioscience, clone: Jes5-16E3) and IL-12 (eBioscience, clone: C17.8). All samples were measured on a FACS Canto II (BD Biosciences)

Quantification and statistical analysis. All statistical analyses were performed using Prism (GraphPad Software Inc). Statistical significance of differences was evaluated with the Student's *t*-test. Significance was accepted at the level of *P* < 0.05.

Chapter 4

Fine tuning cell migration by A Disintegrin And Metalloproteinases

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Abstract

Cell migration is an instrumental process involved in organ development, tissue homeostasis, and various physiological processes and also in numerous pathologies. Both basic cell migration and migration towards chemotactic stimulus consist of changes in cell polarity and cytoskeletal rearrangement, cell detachment from, invasion through, and reattachment to their neighbouring cells, and numerous interactions with the extracellular matrix. The different steps of immune cell, tissue cell, or cancer cell migration are tightly coordinated in time and place by growth factors, cytokines/chemokines, adhesion molecules, and receptors for these ligands. This review describes how a disintegrin and metalloproteinases interfere with several steps of cell migration, either by proteolytic cleavage of such molecules or by functions independent of proteolytic activity.

1. Principles of Cell Migration

Cell migration is a critical step in the homeostatic and inflammatory trafficking of immune cells, the migration of cells during embryogenesis, in regenerative processes such as wound healing, in tissue homeostasis, and also in the development of diseases such as cancer ¹. Dysregulation in migration can result in severe peri- or postnatal defects such as the neural tube defect ², heart abnormalities, and defective lymphopoiesis ^{3,4}. Further, in the adult organism, several pathologies are linked to alterations in migration, including inflammatory disorders such as rheumatoid arthritis and multiple sclerosis, vascular diseases ⁵, in which immune cells promote the inflammatory process ⁶, delay of wound closure, and tumor metastasis formation ⁷. The list of migrating cell types is long differing in their form and speed of migration including immune cells, epithelial cells, endothelial cells, smooth muscle cells, pericytes, and neural cells. The exact mechanisms of cell migration can differ especially between rapidly migrating leukocytes and tissue cells. However, the involved surface molecules, the signal transduction pathways, and the underlying molecular machinery show a considerable degree of overlap in all motile cells.

On the migrating cell itself, a well-orchestrated sequence of single steps can be observed such as polarity changes, protrusion and retraction, and loose and firm adhesion to other cells or the extracellular matrix (ECM). Leukocytes and also cancer cells are capable of transmigrating through the tissue layers including endothelium or epithelium ⁷. This also involves interaction with these tissue layers, which often regulate adhesion and junction molecules, thereby increasing permeability of the cell layer ⁸ as well as transmigration of the migrating cells. The polarizing and initiating stimulus can be of various nature: chemotactic (i.e., chemoattractants and morphogens) ¹; haptotactic (i.e., varying substrate concentrations in wound healing, angiogenesis, and metastasis) ⁹; mechanotactic (i.e., loss of cell-cell contacts in wound healing or metastasis) ¹⁰; durotactic (i.e., varying rigidity) ¹¹. Polarization is accompanied by the extension of generally formed pseudopods towards the direction of migration, driven by the rearrangement of the actin cytoskeleton ¹². The different protrusions mediate the interaction with surrounding tissue cells and the ECM and the formation of adhesive complexes. The presence of nascent adhesions and focal complexes are markers of fast migrating cells, whereas focal adhesions as more mature structures are inversely correlated with cell motility ¹³. The most important common components of adhesive complexes are integrins as adhesion receptors. Integrins are cell specifically expressed and activated upon specific stimulation, thereby mediating leukocyte adhesion and transmigration ¹⁴. Podosomes are found in fast moving cells such as macrophages, sharing similar structures with invadopodia of metastatic tumor cells ¹⁵. Both include the redirection of integrin receptors and adhesion molecules to the leading edge of the migrating cells, while invadopodia further concentrate proteolytic components that degrade the surrounding matrix to facilitate transmigration ¹⁶. Often, tissue or cancer cell migration requires the acquisition of a migratory phenotype. These phenotypic changes can be brought about by cytokines, growth, or differentiation factors. For example, repair processes involving tissue cell migration and also cancer cell migration can be initiated within the tissue layers by transforming growth factor (TGF) and heparin-binding epidermal growth factor (HB-EGF) ¹⁷⁻¹⁹.

One of the most studied migratory events is the recruitment of immune cells from the blood to a site of inflammation, for example, caused by wounding or infection. Pro-inflammatory signals are released and relayed to the vascular endothelium, which exposes new adhesion molecules and receptors (e.g., E-selectin and P-selectin, vascular adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1), CXCL16, and CX3CL1²⁰⁻²³). Immune cells are slowed down in migration and loosely adhering to the endothelium, rolling along the endothelium via the interaction of selectins with glycoprotein ligands, adhere more tightly via activated integrins, crawl on the endothelium probing for an extravasation point, and at last transmigrate through the endothelium. Thus, for effective migration of immune cells, tissue cells or cancer cells several migratory steps need to be tightly coordinated. This involves the regulation of cytokines, growth factors, chemokines, adhesion molecules, and receptors for these ligands. Notably, many of these molecules are expressed as membrane-bound form and are functionally modulated by limited proteolysis close to the plasma membrane, a process called shedding. In many cases, members of the family of a disintegrin and metalloproteinases (ADAMs) mediate these shedding events. By this activity, ADAMs can interfere with several steps of cell migration. In addition, some ADAM family members can also regulate adhesion processes independently of any proteolytic activity.

2. ADAM Proteases

ADAM proteases belong to the class of metalloproteinases, which also comprises the matrix metalloproteinases (MMP) and a disintegrin and metalloproteinase with thrombospondin motif (ADAMTS). The ADAM family consists of 34 members, with 22 members described in humans^{24,25}. Most ADAMs are expressed as type I transmembrane surface proteins with a typical multidomain structure consisting of an N-terminal metalloproteinase domain, a disintegrin domain, a cysteine-rich domain, an EGF-like domain followed by a transmembrane domain, and a cytoplasmic tail (**figure 1**). Most ADAMs are synthesized as proenzymes, in which the N-terminal and inhibiting prodomain is released by furin-mediated cleavage during the maturation process. The zinc-dependent metalloproteinase domain mediates the proteolytic activity, which is only present in ADAM8, ADAM9, ADAM10, ADAM12, ADAM15, ADAM17, ADAM19, ADAM20, ADAM21, ADAM28, ADAM30, and ADAM33²⁶. ADAM2, ADAM7, ADAM11, ADAM18, ADAM22, ADAM23, ADAM29, and ADAM32 lack the consensus sequence HExGHxxGxxHD for metalloproteinase activity. Their function remains largely unknown but may include functions as adhesion molecules rather than as proteases. The cysteine-rich domain and disintegrin domain of most ADAM proteases are involved in substrate recognition and interaction with integrins and the extracellular matrix. ADAM10 and ADAM17 do not carry the typical EGF-like domain. Their cysteine-rich domain is followed by a so-called membrane-proximal domain as well as a small stalk region²⁷⁻²⁹. The cytoplasmic tail is very diverse in length and may be involved in signaling between the intracellular and extracellular portion as well as in assembling of cytoplasmic adaptor molecules. Some ADAM proteases exist not only as transmembrane proteins but also as soluble forms resulting from splice variants. These soluble forms of ADAM9, ADAM12, and ADAM28 lack the transmembrane domain and the C-terminal cytoplasmic tail²⁶.

Figure 1: Catalytic and noncatalytic functions of ADAM proteases facilitating cell migration. ADAM proteases interfere with distinct steps of cell migration via a number different effector molecules. This involves proteolytic cleavage or nonproteolytic interactions of the proteases. ADAMs consist of a C-terminal cytoplasmic tail (CC) followed by a transmembrane domain (TM), an EGF-like domain (EL), a cysteine-rich domain (CR), and a disintegrin domain (D) and an extracellular metalloproteinase domain (MP) (ADAM). ADAM proteases cleave transmembrane surface molecules close to the plasma membrane, a process called shedding, requiring a zinc atom in the active site of the metalloproteinase domain (I). This results in the release of a soluble ectodomain and the production of a cell-associated fragment consisting of the transmembrane and cytoplasmic domain. The remaining fragment can undergo further regulated intramembrane proteolysis by γ -secretase (II) at last functioning as transcription factor (e.g., Notch). ADAM activity can be regulated by activation of a precursor protein (e.g., pro-MMP9). The released ectodomain may function either as agonist like the chemokine CXCL16 and the ErbB ligands HB-EGF and neuregulin or as antagonist like soluble JAM-A or RAGE (III). The release of ErbB ligands leads to transactivation of ErbB receptors in cis and trans (IV). The inactivation of ligands (i.e., adhesion molecules (AM)) can lead to the detachment of bound receptor complexes and attached cells (i.e., L-selectin, MAC-1) (V). The cleavage of junction molecules (JM, i.e., cadherins) results in changes of permeability (VI), facilitating the transmigration of inflammatory cells, the invasion of cancer cells, and the dissemination resulting in metastasis and angiogenic processes (VII). The permeability is further regulated by ECM degradation, by either direct action of ADAM proteases (i.e., ADAM9) or the regulation of MMPs (VIII). With their disintegrin-like domain, ADAMs can directly interact with adhesion molecules and ECM proteins (IX). The integrin (indicated by α/β) interaction/activation results in cytoskeletal rearrangement, focal adhesion formation, and podosome extension (X). Integrin, ADAM activation, and ErbB receptor transactivation result in different signaling pathways including the activation of Rho, Rac, FAK, PI3K, AKT, p38, and ERK, all further regulating ADAM and integrin activity as well as gene expression (XI). The exact mechanism of cell migration can differ between rapidly migrating leukocytes and tissue cells. However, the involved surface molecules, the signal transduction pathways, and the molecular machinery show a considerable degree of overlap for the action of ADAM proteases in inflammatory cell recruitment, angiogenesis, reepithelialization, cancer cell detachment and adhesion, and the intra- and extravasation during metastasis.

Proteolytic activity has been reported for ADAM8, ADAM9, ADAM10, ADAM12, ADAM15, ADAM17, ADAM28, and ADAM33³⁰. The interaction partners and substrates of ADAMs are generally transmembrane surface proteins. It is important to note that, with only one reported exception²⁹, shedding occurs in cis and not in trans, meaning that substrate and protease have to be expressed on the same cell. The regulated proteolysis occurs close to the cell surface, resulting in the release of a soluble ectodomain and the production of a cell-associated fragment consisting of the transmembrane and the cytoplasmic domain. After this shedding process, the remaining fragment can undergo

further regulated intramembrane proteolysis (“RIPping”), which has been reported for Notch ³¹. ADAM activity can convert surface molecules into soluble agonists. By this, ADAMs critically drive the activity of cytokines (e.g., TNF), chemoattractants (e.g., CX3CL1), or growth factors (e.g., EGF). Further, released ectodomains can act as antagonists and sequester soluble ligands (e.g., TNFR). Shedding of a receptor or adhesion molecule (e.g., L-selectin) can lead to reduced responsiveness (e.g., VEGFR2) or cell adhesion (e.g., RAGE, L-selectin). Moreover, proteolytic release of an adhesion molecule bound to its receptor can result in detachment of adherent cells. Finally, cytoplasmic fragments released by RIPping may function as transcription factors (e.g., Notch). Notably, ADAMs can directly interact with adhesion molecules and extracellular matrix proteins (e.g., collagen). This interaction involves the disintegrin domain and can promote cell-to-cell adhesion or strengthen barrier function of cell layers ³².

This broad activity requires a regulatory network to limit the proteases activity in space and time. It is not yet fully understood how the substrate selectivity of the single ADAM proteases is mediated, as the substrates do not seem to carry a distinct cleavage site ^{33,34}. Specificity is possibly mediated by the substrate binding pocket ³⁵, by exosites such as the cysteine-rich domain ²⁹, the juxtamembrane region, and the transmembrane domain ^{27,36-38}. Further, involved signaling pathways ³⁹ as well as changes in the membrane structure ^{40,41} may influence ADAM substrate specificity. Regulation of ADAM protease activity can occur on various levels. Acute and chronic stimulation and the tumor environment have been shown to enhance ADAM protease gene expression ^{32,42,43}. The increase of gene expression and enzyme synthesis are rather slow regulators of shedding activity. Rapid regulation of proteolytic activity occurs on the posttranscriptional level including the removal of the inhibitory prodomain ⁴⁴, changes in membrane distribution ^{41,45}, release and transport to the cell surface ^{46,47}, multimerization on the cell surface ⁴⁸, autocatalytic activation ⁴⁹, interaction with adaptor molecules, and conformational changes ^{40,50,51}. Recently, pseudoproteases of the rhomboid family, the inactive rhomboid (iRHOM) 1 and 2, have been discovered as adapter molecules of ADAM17 in the endoplasmic reticulum mediating the transport of the protease to the Golgi apparatus and the cell surface ^{52,53}. ADAM10 does not seem to interact with iRHOMs, but its surface expression strongly depends on tetraspanins of the TSPANC8 family ^{54,55}. Interestingly, also ADAM17 is regulated by the tetraspanin CD9/TSPAN29 ⁵⁶. Since iRHOMs and tetraspanins are critical for ADAM activity on the cell surface, they very likely also interfere with ADAM-dependent mechanisms of cell migration. Furthermore, tissue inhibitors of metalloproteinases are well known to block ADAM proteases. TIMP-1 turned out to be a relevant inhibitor of ADAM10 ⁵⁷, while TIMP-3 is more effective on ADAM17 ⁵⁸. Thus, regulation of TIMP1 or 3 also represents a means of controlling ADAM10- or ADAM17-mediated migratory processes. As discussed below, rapid posttranslational regulation of shedding events and long-term transcriptional induction of protease activity contribute to very basic migratory events involved in many different pathologies including inflammation, healing responses, and tumor metastasis.

3. ADAM Functions in Leukocyte Migration

As mentioned above, ADAM proteases may regulate cell migration either by their shedding activity or by the noncatalytic functions such as matrix and integrin interactions (Figure 1).

3.1. ADAM-Mediated Shedding in Leukocyte Migration

Leukocyte migration through tissue cells can depend not only on the proteolytic activity of ADAMs on the leukocytes themselves (see Table 1) but also on the ADAM protease activity on the tissue cells serving as substrate of cell migration (see Table 2).

In tissue cells transmigrated by leukocytes, ADAM10 and ADAM17 are the predominant proteases mediating shedding events that regulate leukocyte migration. Both endothelial and epithelial cells form a dense barrier to insure tissue integrity, which has to be opened transiently for transmigration. Upon stimulation with VEGF or thrombin, VE-cadherin is shed from the endothelial cell surface by ADAM10 and ADAM17^{59,60}. The epithelial counterpart E-cadherin is solely shed by ADAM10 under physiological as well as pathological conditions⁶¹. Whereas shedding of VE-cadherin and E-cadherin has been shown to facilitate transmigration, the function of JAM-A is divergent. In its transmembrane form, JAM-A controls endothelial tight junction formation and contributes to leukocyte transendothelial migration^{62,63}. In contrast, soluble JAM-A, predominantly released by ADAM17, was shown to reduce inflammatory cell recruitment both *in vitro* and *in vivo*, avoiding excessive infiltration⁶². Endothelial cells express the transmembrane chemokines CXCL16 and CX3CL1, which are upregulated under inflammatory conditions and constitutively and inducibly shed by ADAM10 and ADAM17^{22,64,65}. The transmembrane forms act as adhesion molecules, regulating the interaction of T lymphocytes and monocytes with tissue cells^{66,67}, whereas the soluble variants act as chemokines, mediating the recruitment of CXCR6 or CX3CR1 expressing cells to the site of inflammation⁶⁸⁻⁷³. In inflammatory diseases, CX3CL1 is released not only by the endothelium but also by monocytes, T lymphocytes, and NK cells as shown for rheumatoid arthritis and cardiovascular diseases⁷⁴ and further enhances the recruitment of inflammatory cells from the circulation.

Meprins A and B, receptor for advanced glycation end products (RAGE), and activated leukocyte cell adhesion molecule (ALCAM (CD166)) are additional substrates shared by leukocytes and tissue cells in leukocyte migration. The meprins are shed by ADAM10^{87,89} releasing a soluble form and facilitating migration through the cleavage of ECM molecules⁸⁸. Additionally, meprin B cleaves E-cadherin, enhancing the induction of epithelial permeability¹²¹, which could be negatively regulated by ADAM10. Trauma or inflammatory stimulation leads to the upregulation of RAGE on epithelial and endothelial cells, facilitating the adhesion and subsequent diapedesis of leukocytes^{132,133}. The function of RAGE on leukocytes appears to be cell-type dependent. For macrophages, dendritic cells and T cells' influences only on activation, differentiation, and proliferation were reported. However, on neutrophils, RAGE was described as a chemotactic receptor interacting with high mobility box 1 protein (HMGB1)⁹¹.

Table 1: Catalytic and non-catalytic functions of leukocytic ADAM proteases in cell migration

Protease	Domain	Cell type	Substrate or interaction partner	Process	References
ADAM7	D	lymphocytes	$\alpha 4\beta 7$, $\alpha 9\beta 1$, $\alpha 4\beta 1$	adhesion	75
ADAM8	?	T cells, eosinophils, dendritic cells, monocytes	?	migration	76-78
un	?	leukocytes	αL upregulation	migration	unpublished
ADAM9	MP	leukocytes	PSGL-1	adhesion, migration	79,80
ADAM10	D	neutrophils	$\alpha v\beta 3$; $\alpha 9\beta 1$	migration	81
	MP	myeloid cells, hematopoietic stem cells	c-met	migration (ECM degradation)	82
	MP	neutrophils	CD44	migration (ECM interaction)	83
	indirect; MP	mast cells	Collagen IV	SCF-induced migration	84
	MP	monocytes	CX3CL1	chemotaxis	74
	MP	neutrophils/monocytes/lymphocytes	DDR1	migration (pseudopod extension)	85
	MP	neutrophils/monocytes	EGFR ligands	migration	86
	MP	monocytes	Meprin A	migration (ECM degradation)	87,88
	MP	monocytes	Meprin B	migration (ECM degradation)	89
	MP	neutrophils	RAGE	negative regulation of adhesion/spreading/migration	90,91
	MP	leukocytes	$\alpha 5\beta 1$, basement membran interaction, cytoskeletal rearrangement	adhesion and migration	86,92
ADAM17	MP	monocytes, lymphocytes	ALCAM	reduced extravasation (loss of adhesion)	93-95
	MP	neutrophils	CD44	migration (ECM interaction)	83
	MP	monocytes	CX3CL1	chemotaxis	74
	MP	neutrophils	CXCR2	reduction of recruitment	96
	MP	B-lymphocytes	Jagged-1/Delta-like4/Notch	migration	97
	MP	neutrophils	L-selectin	rolling, reduction of transmigration (higher velocity, reduced adhesion), resolution of transmigration	98-100
	MP	monocytes	Mac-1	end of transmigration	101
	MP	Langerhans cells, lymphocytes	TNFR1	migration	102,103
ADAM28	D	lymphocytes	$\alpha 4\beta 7$; $\alpha 9\beta 1$; $\alpha 4\beta 1$	adhesion	75
ADAM33	D	lymphocytes	$\alpha 9\beta 1$	adhesion	75

The process indicates the function in the presence of the ADAM protease. MP: Metalloproteinase domain; D: Disintegrin-like domain; ? : unknown

Table 2: Catalytic and non-catalytic functions of tissue ADAM proteases in cell migration

Protease	Domain	Cell type	Substrate or interaction partner	Process	Reference
ADAM8	D, CR	endothelial cells	PECAM-1, isolectin B4, Tie2, VEGFR1/2, EphB2, VE-cadherin	angiogenesis	104,105
ADAM9	MP	keratinocytes	collagen XVII, α3β1a, MMP expression	reduced migration/wound healing	106-108
	MP	epithelial cells	EGFR ligands	migration	109
	MP	vascular smooth muscle cells	HBEGF	migration	110
	D, CR	fibroblasts	α3β1, α5β1, α6β1	cell-cell-interaction and ECM degrading capacity	108
ADAM10	MP	epithelial cells	betacellulin	migration	111
	MP	trophoblasts	c-met	block of invasion	112
	MP	glial precursor cells	CXCL16	migration, invasion	71
	MP	mesangial cells	CXCL16	migration	113
	MP	endothelial cells	CXCL16, CX3CL1	chemotaxis, migration, transmigration	22,64,67,114,115
	?	fibroblast-like synoviocytes	cytoskeletal rearrangement, VEGFA, MMP1- and MMP3 expression, CX3CL1	migration	73,116
	MP	epithelial cells	DDR1	collagen-adhesion, migration	117
	MP	vascular smooth muscle cells	DDR1	migration	118
	MP	epithelial cells	E-cadherin	transmigration, keratinocyte migration	61
	MP	neural cells	L1CAM	migration	119,120
	MP	endothelial cells, epithelial cells	Meprin A, Meprin B	transmigration (ECM degradation)	87-89,121
	MP	neural progenitor cells	N-cadherin	migration	122
MP, ?	MP	vascular smooth muscle cells	N-cadherin, β-catenin	migration	123
	MP	endometrial cells	neuregulins	migration	125
	MP	border cells	Notch	limitation of vascular sprouting	126-129
	MP	endothelial cells	Notch	migration	130
	MP	vascular smooth muscle cells	Notch 1, Notch 3	migration	131
	MP	pericytes, smooth muscle cells	PDGFRβ	block of transmigration	90,132,133
	MP	endothelial cells, epithelial cells	RAGE	transmigration	59,60
	MP	endothelial cells	VE-cadherin	limitation of sprouting	60,134
	MP	endothelial cells	VEGFR2	cell invasion placental development	135
ADAM12	MP	trophoblasts	?	migration	136
	MP	keratinocytes	HB-EGF, IGF	limitation of sprouting	137
	MP	endothelial cells	VEGFR2	limitation of sprouting	?

The process indicates the function in the presence of the ADAM protease. MP: Metalloproteinase domain; D: Disintegrin-like domain; CR: cysteine-rich; ?: unknown

Table 2 continued: Catalytic and non-catalytic functions of tissue ADAM proteases in cell migration

Protease	Domain	Cell type	Substrate or interaction partner	Process	Reference
ADAM13	MP	cranial neural crest	cadherin-11	migration (avoidance of contact inhibition)	138
	MP	mesenchymal progenitors	amphiregulin	migration	150
	MP	trophoblasts	c-met	block of invasion	112
	MP	endothelial cells	CX3CL1	transmigration, chemotaxis	67,151
	MP	mesangial cells	CXCL16	migration	113
ADAM15	MP	endothelial cells	HB-EGF	migration	17,152
	?	intestinal epithelial cells	?	inhibition of migration (wound healing)	139
	MP	glomerular mesangial cells	collagen IV, gelatin, fibronectin	migration (matrix degradation)	140
	?	fibroblast-like synoviocytes	VEGFA-, MMP1- and MMP3 expression	migration	141
ADAM17 ADAM17	D, ?	endothelial cells	$\alpha 5\beta 1$, $\alpha v\beta 3$, Src activation, ?	angiogenesis, transmigration, adhesion, migration	142-148
	D	airway smooth muscle cells	$\beta 1$	block of transmigration	149
	MP	endothelial cells	ALCAM	migration	93-95
	MP	endometrial cells	amphiregulin	block of transmigration	124
	MP	mesenchymal progenitors	amphiregulin	migration	150
	MP	trophoblasts	c-met	block of invasion	112
	MP	endothelial cells	CX3CL1	transmigration, chemotaxis	67,151
	MP	mesangial cells	CXCL16	migration	113
	MP	endothelial cells	HB-EGF	migration	17,152
	MP	endothelial cells	JAM-A	block of leukocyte transmigration and endothelial migration	62,63
	MP	neural progenitor cells	L1-CAM, HB-EGF	migration	119,153
	MP	pericytes, smooth muscle cells	PDGFR β -EGFR crosstalk	migration	131
MP	MP	epithelial cells	syndecan-1	migration	154,155
	MP	epithelial cells (e.g. keratinocytes)	TGF α , amphiregulin, epigen, VEGFR	migration	17,152,156
	MP	endothelial cells	TNF α , TNFR	time-dependent regulation of angiogenesis	157-160
	MP	endothelial cells	TNFR	migration	161
	MP	endothelial cells	VE-cadherin	transmigration	17,60
	MP	endothelial cells	VEGFR2	angiogenesis	162
	MP	endothelial cells			
	MP	endothelial cells			
	MP	endothelial cells			
	MP	endothelial cells			

The process indicates the function in the presence of the ADAM protease. MP: Metalloproteinase domain; D: Disintegrin-like domain; CR: cysteine-rich; ?: unknown

It is important to note that, in contrast to transmembrane RAGE, soluble RAGE released by ADAM10 functions as negative regulator of adhesion, blocking the activation of MAPK3 and PI3K. This inhibition of spreading and migration⁹⁰ could represent a mechanism for fine tuning of inflammatory cell recruitment. A similar function has been reported for soluble ALCAM, which may prevent the accumulation of premature tissue macrophages⁹⁵. ALCAM is expressed on endothelial cells and upregulated upon inflammatory stimulation. Under homeostasis, peripheral blood monocytes and lymphocytes show only weak expression of ALCAM, whereas inflammation leads to upregulation of surface ALCAM, enabling homophilic (ALCAM-ALCAM) or heterophilic (ALCAM-CD6) interactions and supporting transmigration⁹³⁻⁹⁵. Especially on T lymphocytes, ADAM17-mediated ALCAM shedding appears to be essential for activated lymphocyte recruitment⁹⁴. Clustering of ALCAM via upregulation of the tetraspanin CD9 was found to limit ADAM17-mediated shedding and lymphocyte recruitment⁹⁴.

Also on leukocytes themselves, ADAM10 and ADAM17 are the predominant shedding enzymes, influencing cell-cell and cell-matrix interactions. Mac-1, which is expressed on monocytes, is one of the major interaction partners for endothelial expressed RAGE. Although Mac-1 cleavage by ADAM10 and ADAM17 was shown, only spatial and temporal ADAM17-dependent cleavage is essential for transendothelial migration of monocytic cells¹⁰¹. The function of L-selectin as adhesion molecule and interaction partner of E-selectin⁹⁸ has been extensively studied in neutrophil migration. L-selectin shedding by ADAM17 limits recruitment of neutrophils at early time points during inflammation. This is mediated by weakening of adhesion during leukocyte rolling along the endothelium leading to higher velocity of rolling leukocytes. In contrast to neutrophils, monocyte recruitment is not affected by L-selectin shedding¹⁰⁰. ADAM17 mediated proteolytic shedding has been reported for CXCR2, the receptor for CXCL1 and CXCL8. Stimulation by lipopolysaccharide (LPS) or formyl peptide leads to enhanced CXCR2 shedding via ADAM17 and impairs neutrophil recruitment, which can be detrimental during excessive inflammation⁹⁶. ADAM10 and ADAM17 are both responsible for the cleavage of epidermal growth factor receptor (EGFR) ligands, resulting in transactivation of EGFR¹¹¹. However, for leukocyte migration, a distinct ligand has not been identified so far⁸⁶. The migration of B lymphocytes towards Notch ligands was shown to depend on ADAM17 and γ -secretase activity, indicating a role of Notch receptor shedding in B cell migration⁹⁷. It is not yet clear whether this could also account for other leukocyte populations. Involvement of cleavage events in the interaction with extracellular matrix proteins has been reported for CD44, the discoidin domain receptor 1 (DDR1), and the hepatocyte growth factor (HGF) receptor c-met. CD44 interacts with hyaluronic acid and is stimulus-dependently cleaved by either ADAM10 or ADAM17, involving Ca²⁺ influx or PKC and Rac activation¹⁶³. Proteolysis of CD44 is essential for leukocyte migration as was exemplarily shown in liver neutrophil trafficking⁸³. DDR1 is expressed on neutrophils, monocytes, and lymphocytes and mediates the adhesion to collagen. DDR1-collagen interaction results in DDR1 phosphorylation and subsequent shedding by ADAM10 allowing pseudopod extension and migration through p38 and NF- κ B activation⁸⁵. c-met surface expression and signaling are regulated by ADAM10- and ADAM17-mediated shedding. Migration of G-CSF mobilized myeloid cells and hematopoietic stem cells was shown to be in parts mediated via the HGF/c-met axis,

increasing matrix degradation by enhancement of MMP expression⁸². In addition, cytokines can promote the migratory response of leukocytes. For example, migration of Langerhans cells in the skin and the mucosa largely depends on IL-1 and TNF α ¹⁰². Also, for IL6, a promigratory function on T cells has been described¹⁶⁴. This would imply that proteolytic shedding of cytokines like TNF α also regulates immune cell migration, and this activity could be counterregulated by receptor shedding of TNFR, IL1R, or IL6R^{102,165,166}. However, linking investigations are still missing.

Besides ADAM10 and ADAM17, only ADAM8 has been shown to influence leukocyte migration by catalytic activity. The P-selectin glycoprotein ligand-1 (PSGL-1) mediates leukocyte rolling on the vascular endothelium in inflammation and is further essential for the migration of T lymphocytes under homeostatic conditions. ADAM8-dependent cleavage at the uropod loosens the initial binding and facilitates leukocyte migration^{79,80,167}. However, besides ADAM8, also ADAM10 and the aspartyl protease BACE1 have been reported to cleave PSGL-1¹⁶⁸, and the relative contribution of each protease remains unclear.

3.2. ADAM Noncatalytic Functions in Leukocyte Migration

In addition to the proteolytic shedding, many ADAMs display noncatalytic functions that can contribute to leukocyte migration. Such activity has been reported for ADAM8, ADAM9, ADAM10, ADAM15, ADAM17, ADAM19, ADAM28, and ADAM33. Most of these functions involve the regulation of integrins, directly affecting integrin-dependent adhesion as one of the first steps in leukocyte recruitment. Adhesive contacts via integrins are required for cell motility, but too tight adhesions prevent cell migration. Regulation of integrins can be a direct result of integrin binding to noncatalytic domains of ADAMs or result from the activation of signaling pathways leading to modulation of integrin expression and activity at the cell surface.

ADAM8 is essential for the clustering of β 1 integrin at the leading edge of migrating cells⁴⁸. However, the function of this mechanism was not yet shown for leukocytic cells, but a promigratory/proasthmatic as well as antimigratory/antiasthmatic function of ADAM8 was reported in ovalbumin induced asthma^{76,77}. ADAM15 is expressed on endothelial cells and enhanced during inflammatory processes, but circulating monocytes lack ADAM15 expression¹⁴⁴. ADAM15 surface expression correlates with expression of α 5 integrin, thereby influencing migration through adhesion¹⁴⁸. Furthermore, the cytoplasmic tail of ADAM15 induces signaling processes including ERK1/2 and Src phosphorylation, which enhance endothelial permeability and monocyte transmigration^{143,144}. ADAM28 in Jurkat cells can support α 4 β 1, α 4 β 7, α 9 β 1 and dependent adhesion, whereas ADAM33 can only interact with α 9 β 1¹⁶⁹. ADAM9 was shown to promote neutrophil activation and chemotaxis by engagement of α v β 3 and α 9 β 1 integrins within a hierarchical cross talk of integrins with CXCR2, involving GPCR, PI3K/AKT, and MAPK activation⁸¹. In myeloid cells, ADAM10 deficiency correlated with reduced surface upregulation of α 5 β 1 integrin upon CCL-2 stimulation accompanied by impairment of p38 and Rho activation, cell-matrix interaction, and cytoskeletal rearrangement, resulting in inhibition of migration

⁸⁶.

4. Migratory Function of ADAM Proteases in Inflammatory Diseases

The role of ADAMs in inflammatory diseases is still not fully understood. As described above, leukocytes and tissue cells express various ADAMs, which are likely to modulate cell migratory processes involved in the pathogenesis of many inflammatory diseases. As examples, we here briefly mention multiple sclerosis (MS), rheumatoid arthritis (AR), and atherosclerosis. We will here focus on the impact of ADAMs in cell migration. Other ADAM-dependent pathogenic mechanisms are reviewed elsewhere¹⁷⁰.

It was clearly shown that MMPs and TIMPs hold promigratory functions in MS, contributing to the pathogenesis of the disease¹⁷¹. However, ADAM proteases can be only indirectly linked to migratory processes so far. Investigation of postmortem brain sections of MS patients revealed that ADAM10 is expressed in astrocytes and sometimes in perivascular macrophages¹⁷². On the other hand, ADAM17 expression is associated with active MS lesions and gadolinium-enhancing lesions of relapsing-remitting MS patients¹⁷³, more specifically in blood vessel endothelial cells, activated macrophages, microglia, astrocytes¹⁷⁴, and invading T lymphocytes¹⁷². Invading T lymphocytes are one major source of TNF, which is shed by ADAM17 and associated with the relapse phase but would rather indirectly contribute to migratory processes. Further, CX3CL1, which is found in the brain and serum during MS, could fulfill different functions. On the one hand, CX3CL1 could activate and recruit circulating leukocytes, but, on the other hand, the adhesion to membrane-bound CX3CL1 could be blocked by its soluble form¹⁵¹.

ADAM8, ADAM10, ADAM15, and ADAM17 have been shown to be upregulated in synovial fluid or tissue of RA patients compared to healthy individuals^{47,73,175,176}. ADAM17 is responsible for the strong TNF release, which orchestrates the inflammatory response in RA. However, the TNF α release influences the migratory process only indirectly. Expression levels of ADAM8 and ADAM10 in the synovial fluid correlate with the degree of joint inflammation and disease severity, respectively^{47,73}. ADAM10 knockdown in RA reduced the LPS-induced migration and invasion of fibroblast-like synoviocytes (FLS) *in vitro*¹¹⁶. ADAM10 was clearly enhanced in biological fluids of RA patients, displaying a higher monocyte migratory activity⁷³. Further, ADAM10 plays a proangiogenic role in RA indicated by an elevated tube formation of endothelial cells, which may be due CX3CL1 and vascular endothelial growth factor (VEGF) release¹¹⁵. One possible treatment of RA is tocilizumab, an antibody against the IL-6R. ADAM10 has been shown to be the major sheddase involved in induced shedding of IL-6R in human¹⁷⁷. ADAM10 was shown to correlate with soluble CX3CL1, which was reduced upon antibody treatment and could serve as predictive marker¹¹⁴. However, the orchestration of the events and the contribution to cell migration of endothelial cells, FLS, and leukocytes in RA have still to be examined. Similar to ADAM10, knockdown of ADAM15 results in suppressed migration and invasion of FLS and silencing of ADAM15 in a rat model of CIA reduced the arthritis score and the extent of joint damage¹⁴¹. It was further shown that VEGF treatment enhances ADAM15 expression in synovial fibroblasts and in endothelial cells, if VEGFR2 expression was increased by TNF α stimulation. The overexpression of ADAM15 in RA synovium and the regulation through VEGF and VEGFR2 suggest a possible function of ADAM15 in angiogenesis in RA synovium¹⁴⁶.

ADAMs 8, 9, 10, 12, 15, 17, and 33 are expressed in human atherosclerotic lesions¹⁷⁸⁻¹⁸⁰; however, their causal role in the pathogenesis still remains ill-defined. Influences on involved migratory processes have been described only for ADAM10 and ADAM15 so far. The expression of ADAM10 in atherosclerotic lesions has been associated with human plaque progression and neovascularization^{60,179,181}. Genetic ablation of ADAM10 or its inhibition reduces migration of endothelial cells, monocytes, macrophages, and vascular smooth muscle cells *in vitro*^{60,92,123}. Further, the proatherosclerotic effect of MMP8 deficiency indicated by higher neointima formation was shown to be dependent on an ADAM10-, N-cadherin, and β -catenin-mediated pathway¹²³. In addition, myeloid ADAM10 deficiency reduced expression of MMP9 and MMP13 as well as MMP2 gelatinase activity in macrophages. Further, ADAM10-deficient macrophages displayed a more anti-inflammatory phenotype with reduced migratory potential and less production of inflammatory mediators like TNF α , IL-12, and NO upon LPS stimulation. These effects together led to higher plaque collagen content, resulting in a shift from inflammation to fibrosis in atherosclerosis due to altered ECM degradation and a shift in atherosclerotic plaque stability⁹². Furthermore, ADAM15 deficiency reduced atherosclerotic lesion progression in mice due to improved endothelial barrier function and reduced monocyte transmigration via Src activation¹⁴⁴.

5. ADAM Proteases in Cell Migration during Healing Responses

Wound healing is a highly dynamic process that involves the coordinated response of a considerable number of different cell lineages in an attempt to restore tissue integrity and homeostasis and is fundamentally similar among tissue types. The wound healing process is characterized by overlapping phases of inflammation, angiogenesis, reepithelialization, and resolution¹⁸². These steps involve proliferative as well as migratory responses of various tissue cells and leukocytes. The role of ADAMs in leukocyte recruitment has been discussed in the previous section. For the purpose of this review, we will focus on ADAM-dependent migration in angiogenesis and reepithelialization in this section (see Table 2).

5.1. Angiogenesis

During wound healing, the angiogenic process is initiated, which involves sprouting of wound edge capillaries followed by invasion into the site of damage. After a few days, the microvascular network is apparent throughout the wound. Sprouting angiogenesis is a multistep process involving endothelial cell proliferation and guided migratory invasion, which are regulated by proteolytic and nonproteolytic activities of ADAMs¹⁸³. Endothelial cells in sprouts are guided by tip cells followed by a multicellular stalk of endothelial cells, which are interconnected by VE-cadherin at cell-cell junctions and successfully form an inner lumen. One of the best studied proangiogenic signals, namely, VEGF, controls the directed migration of the tip cells¹⁸⁴. ADAM10, ADAM12, and ADAM17 have been shown to shed VEGFR2 upon activation *in vitro*, thereby limiting vascular sprouting^{60,134,137}. However, ADAM10 and ADAM17 can have opposing effects

on physiological angiogenesis *in vivo*. While genetic ablation or inhibition of ADAM10 increases vascular sprouting, most likely due to reduced shedding-mediated Notch activation, ADAM17 hypomorphic mice or inhibition of ADAM17 decreases angiogenesis *in vivo*^{127-129,185}. Interestingly, ADAM17 may exert both positive and negative effects on angiogenesis by timely release of TNF α ¹⁵⁷ or its receptor TNFR. TNF α stimulation increased ADAM17 expression accompanied by increased shedding of TNFR, which could be a self-protective mechanism during prolonged immunostimulation¹⁵⁸. Chronic exposure to TNF α inhibited angiogenesis, while a 2-3-day pulse stimulated angiogenesis by inducing a migratory tip-cell phenotype¹⁵⁹. The different functions of TNF α signaling in ischemia-mediated angiogenesis could be in part explained by the opposite effects of TNFR1 and TNFR2. Stimulation of TNFR2 enhances endothelial cell migration, whereas TNFR1 inhibits migration¹⁶⁰. However, it remains elusive how and when ADAM17 acts regulatory by the shedding of TNFRs in this process. Knockdown of ADAM17 reduces the amount of invading endothelial cells as well as the distance they covered *in vitro*¹⁸⁶. Additionally, the migration of VEGF-A- or FGF7-stimulated endothelial cells depends on EGFR activation via shedding of HB-EGF by ADAM17^{17,152}. Migrating endothelial cells continuously extend and retract processes at the leading edge of the sprout¹⁸⁷. Expression of a dominant negative mutant of ADAM17 increased the number of extending processes in endothelial cells, suggesting that ADAM17 is involved in proper retraction of these peripheral processes¹⁸⁶. During angiogenesis, pericytes and smooth muscle cells are recruited to endothelial cells to stabilize developing blood vessels. This migratory process is mediated via the interaction of platelet-derived growth factor- (PDGF-) B and its receptor PDGFR β . PDGFR β is constitutively shed by ADAM10, whereas ligand-dependent activation does not lead to its own shedding but to stimulation of ADAM17-dependent EGFR ligand shedding. This PDGFR β -EGFR-signaling axis was shown to be critical for PDGF-B stimulated cell migration¹³¹. Not only PDGFR β but also DDR1 and its shedding may be important for angiogenesis. It was shown that DDR is critical in the regulation of attachment to collagen, chemotaxis, and MMP production in smooth muscle cells. Further, DDR1-null mice displayed lower intimal thickening after vascular injury¹¹⁸. As already mentioned before, DDR1 is shed by ADAM10 upon interaction with collagen⁸⁵. Thus, it seems feasible that shedding of DDR1 by ADAM10 could be involved in smooth muscle cell migration and intimal thickening. However, this possibility still has to be investigated. Smooth muscle cell migration plays an important role in artery in-stent restenosis. Studies in minipigs and arterial smooth muscle cells indicated an involvement of ADAM10 in this process, partly mediated by Notch1 and Notch3 cleavage, resulting in nuclear translocation and downstream gene induction¹³⁰.

Both ADAM8 and ADAM15 are also implicated in angiogenesis. Overexpression of ADAM8 *in vitro* increases shedding of coexpressed proteins, including Tie2, VEGFR1 and VEGFR2, EphB2, and junctional molecules CD31 and VE-cadherin¹⁰⁵. Further, ADAM8 was shown to be associated with angiogenesis after spinal cord injury in mice¹⁰⁴. The expression of ADAM15 is upregulated in endothelial cells during angiogenesis, where it can interact with $\alpha 5\beta 1$ and $\alpha v\beta 3$ via its RGD motif¹⁴⁵, while ADAM15 knockout mice showed reduced angiogenic responses to hypoxia¹⁴².

5.2. Reepithelialization

Upon injury, the regrowth of epithelium over a damaged area is pivotal in the repair phase of wound healing. Epithelial cells at the edge of the wounded tissue are instructed to loosen their cell-cell and cell-ECM contacts and migrate across the denuded area, while epithelial cells behind the leading edge start to proliferate until a new epithelium covers the damaged tissue¹⁸².

E-cadherin, an adhesion molecule responsible for anchoring neighbouring cells to one another, is cleaved by ADAM10, thereby decreasing cell-cell contacts and increasing migration of keratinocytes⁶¹. Studies have reported that ADAM9, ADAM10, and ADAM15 are able to cleave ECM substrates *in vitro*, including fibronectin, type IV collagen, and gelatin, thereby facilitating cell migration and release of sequestered growth factors to ECM proteins^{140,188,189}. Shedding of collagen XVII from keratinocytes by ADAM9, ADAM10, and ADAM17 reduced migration due to the inhibitory effect of soluble collagen XVII on migration¹⁰⁶. In line, wound repair was accelerated in ADAM9 deficient mice due to increased migration of keratinocytes and reduced constitutive shedding of collagen XVII¹⁰⁷. Additionally, binding of ADAM9 to the $\alpha 3\beta 1$ integrin in keratinocytes increased MMP9 expression and subsequent cell migration¹⁰⁸. EGFR ligands, including TGF- α , HB-EGF, amphiregulin, betacellulin, and epiregulin, are expressed by keratinocytes and are key regulators of their migration¹⁹⁰, which can be processed into active soluble factors by ADAM9, ADAM10, and ADAM17^{17,109,111,156}. Deficiency of iRHOM2, which is critical for the maturation of ADAM17 in keratinocytes, reduces EGFR-dependent keratinocyte migration underlining the importance of ADAM17 for this response³⁸. Besides the generation of soluble promigratory ligands, ADAM17 may also interfere with reepithelialization by the shedding of TNFR1 and TNFR2. Physiological levels of TNF α enhanced migration through TNFR2, whereas pathological levels inhibited wound closure through TNFR1¹⁶¹. However, as already mentioned studies directly linking ADAM17, TNFR1 and migration are missing. Skin explants from ADAM12 knockout mice displayed increased migration of keratinocytes, potentially via shedding of HB-EGF and insulin-like growth factor (IGF)¹³⁶. In contrast, wound healing in ADAM15 deficient mice was unaltered making ADAM15 dispensable for this process¹⁹¹.

6. ADAM Proteases in Cancer Cell Migration

Tumor metastasis, the dissemination of cancer cells and subsequent outgrowth of secondary tumors at distant sites, is a major contributor to cancer morbidity and mortality. Metastasis is a multistep process involving the detachment of cancer cells from their surrounding tissue, the migration or invasion through the local extracellular matrix towards lymphatic or blood vasculature, the intravasation into the circulation, and lastly the extravasation at a distant tissue to establish a secondary tumor ¹⁹². A number of ADAM proteins are (over)expressed in malignant tumors and have been shown to regulate several steps of the metastatic process, which will be discussed below (see **Table 3**).

6.1. Detachment and Invasion of Cancer Cells

The initial process of tumor invasion involves loss of cell-cell adhesion and increased cellular mobility, resembling many features of epithelial to mesenchymal transition program during developmental processes (e.g., loss of E-cadherin expression, increased expression of N-cadherin, and enhanced growth factor signaling) ¹⁹³. E-cadherin has been shown to be cleaved by ADAM9, ADAM10, and ADAM15 in cancer cells *in vitro* ^{61,194-196}. The cytoplasmic domains of cadherins interact with β -catenin, and reduced surface expression of E-cadherin causes β -catenin to dissociate from the plasma membrane. When stabilized by active Wnt signaling or by mutations in the β -catenin phosphorylation/degradation pathway, β -catenin translocates to the nucleus where it modulates expression of genes involved in cell migration and invasion ¹⁹⁷. Additionally, soluble E-cadherin can disrupt adherens junctions and increase migration and invasion by activating EGFR signaling and upregulating several MMPs ^{196,198}. ADAM10 is a major protease for N-cadherin shedding *in vivo* ²³⁵. Increased N-cadherin expression enhances the migration and invasiveness of cancer cells by acquiring an affinity for mesenchymal stroma cells ^{193,236}. Surprisingly, ADAM10-mediated cleavage of N-cadherin enhances the migration of glioblastoma cells *in vitro* ²¹³. Tetraspanins have been recognized as positive and negative regulators of tumor cell migration ²³⁷. As described above, tetraspanin CD9/TSPAN28 acts as adapter molecule of ADAM17 and contributes to ADAM17 surface activity. By this, CD9 indirectly contributes to shedding of adhesion molecules such as ALCAM, which then may contribute to its reported tumor suppressive function ²³⁷. TSPANC8 family proteins (TSPAN5, TSPAN10, TSPAN14, TSPAN15, TSPAN17, and TSPAN33) differentially regulate ADAM10 compartmentalization and thereby selectively promote cleavage of N-cadherin or CD44 which may represent critical steps in tumor cell migration ^{55,238}. In addition, tetraspanin TM4SF3/TSPAN8 promotes ADAM12 upregulation and by this esophageal carcinoma cell migration ²³⁹.

Similar to keratinocytes, EGFR ligand shedding and subsequent EGFR signaling are a key factor in tumor migration and invasion ^{240,241}. For instance, ADAM17 enhances migration and invasion of breast cancer and glioblastoma stem cells by promoting EGFR signaling through shedding of several EGFR ligands ^{230,231,242} and PI3K/AKT activation, for example, shown for TGF- α shedding in triple-negative breast cancer cell invasion ²³⁰ and EGFR activation in hypoxia-induced glioma cell invasion ²⁴³. ADAM17 generates a transmembrane C-terminal fragment (tCTF) of syndecan-1, which is further processed

Table 3: Catalytic and non-catalytic functions of cancer cell ADAM proteases in cell migration

Protease	Domain	Cell type	Substrate or interaction partner	Process	Reference
ADAM8	D	TNBC, pancreatic cancer	$\beta 1$, increase of MMP activity	Invasion, metastasis	48,199
ADAM8	?	glioma cells	?	invasion	200
L-ADAM9	D	breast cancer	?	Inhibition of migration	201
S-ADAM9	MP	breast cancer	?	migration	201
ADAM9	MP	pancreatic cancer cells, esophageal squamous cell carcinoma	E-cadherin	invasion, metastasis	194
	D, CR	melanoma cells	$\alpha 3\beta 1$, $\alpha 6\beta 1$, MMP upregulation	cell-cell-interaction and ECM degrading capacity, invasion	108
	D	prostate cancer	$\alpha 6\beta 1$	inhibition of migration and invasion	202
	D, CR	colon carcinoma, hepatic stellate and breast cancer cells	$\alpha 6\beta 4$, $\alpha 2\beta 1$, $\alpha 6\beta 1$	liver metastasis	203
ADAM10	MP	pituitary adeno carcinoma, glioblastoma, tumor cells in general	CD44	cell-contact-inhibition, metastasis	163,204,205
	MP	ovarian cancer cells, prostate cancer cells	CXCL16, $\alpha 9\beta 3$	migration, metastasis, attraction of cancer cells	206-208
	MP	esophageal squamous cell carcinoma, breast cancer, pancreatic cancer	E-cadherin, cadherin-catenin-interaction	cell-cell-contact, invasion, migration	195,209
	MP	pituitary adeno carcinoma, glioma	L1CAM	migration	120,210-212
	MP	glioblastoma	N-cadherin, cytoskeletal rearrangement, RAGE	migration	90,213
	MP	melanoma, gastric cancer cells, non small cell lung cancer	Notch1	migration and invasion	214-216
	?	hepatocellular carcinoma	PI3K/AKT activation	invasion	217
	D	glioblastoma sphere-forming cells	$\alpha 5\beta 1$	inhibition metastasis	218
ADAM12	D	oral squamous cell carcinoma	$\alpha 9\beta 6$, MMP regulation	migration and invasion	219
	?	breast cancer cells	?	invasion, metastasis	220
	D, CR	melanoma cells	$\alpha 9\beta 1$	migration	221
	MP	lung tumor cells	Ephrin A1	extravasation	222
ADAM15	MP	breast cancer cells	E-cadherin	migration	223
	MP	non small lung cancer	pro-MMP9	migration (ECM degradation)	224
	MP	breast cancer cells	HB-EGF	migration	225
	D	ovarian cancer cells	$\alpha 9\beta 3$	decrease of migration	226
	MP	prostate cancer cells	?	block of extravasation and bone metastasis	223

The process indicates the function in the presence of the ADAM protease. MP: Metalloproteinase domain; D: Disintegrin-like domain; CR: cysteine-rich; ?: unknown

Table 3 continued: Catalytic and non-catalytic functions of cancer cell ADAM proteases in cell migration

Protease	Domain	Cell type	Substrate or interaction partner	Process	Reference
ADAM17	MP	ovarian carcinoma, thyroid tumors	ALCAM	invasion (loss of adhesion), metastasis	227
	MP	adenoma cells	CD44	invasion	163
	MP	breast cancer, glioblastoma	EGFR ligands	invasion (loss of adhesion)	228-231
	MP	nasopharyngeal carcinoma	EGFR ligands, E-cadherin	invasion	232
	MP	gastric cancer cells, liver cancer	Notch1	migration	215
ADAM17	MP	colon carcinoma	PTK7	migration	233
	MP	lung cancer cells	syndecan-1	metastasis, invasion	154,155
	?	glioblastoma sphere-forming cells	$\alpha 5\beta 1$	inhibition metastasis	218
ADAM19	MP	retinoblastoma cells, non-small-cell lung cancer, glioma	?	invasion, metastasis	200
ADAM23	MP	breast cancer	$\alpha v\beta 3$	metastasis	234

The process indicates the function in the presence of the ADAM protease. MP: Metalloproteinase domain; D: Disintegrin-like domain; CR: cysteine-rich; ?: unknown

by γ -secretase, resulting in a cytoplasmic C-terminal fragment (cCTF). Full-length syndecan-1 and the tCTF mediated lung cancer cell invasion and metastasis, whereas the cCTF antagonized these functions^{154,155}.

Overexpression of ADAM8 in pancreatic ductal adenocarcinoma cells increased their migratory and invasive capacities *in vitro* and *in vivo* by enhancing ERK1/2 signaling and MMP activity and the clustering of $\beta 1$ integrin⁴⁸. Moreover, lung cancer cells express an alternatively spliced variant of ADAM8, termed Delta14, which increased lung metastasis to bone *in vivo*²⁴⁴.

Hepatic stellate cells secrete an alternatively spliced soluble isoform of ADAM9 (ADAM9-S), which can interact with $\alpha 6\beta 4$ and $\alpha 2\beta 1$ integrins on colon carcinoma cells via its disintegrin domain. A similar interaction was also shown for $\alpha 6\beta 1$ integrin in prostate tumor cells²⁰². Integrin-bound ADAM9-S increases the invasive capacity of colon carcinoma cells by cleaving laminin and other ECM components *in vitro*²⁰³. Additionally, ADAM9-S increases breast cancer migration via its metalloproteinase activity, while the transmembrane form of ADAM9 (ADAM9-L) has the opposite effect on migration, which requires its disintegrin domain²⁰¹. Furthermore, ADAM9 disintegrin domain mediates the interaction of fibroblasts and melanoma cells contributing to proteolytic activities required during invasion of melanoma cells²⁴⁵.

ADAM10 can also generate soluble L1 cell adhesion molecule (L1CAM), which stimulates the migration of tumor cells through binding to the $\alpha v\beta 5$ integrin¹²⁰. Additionally, L1CAM shedding can take place either at the cell surface or in the endosomal and exosomal compartments, which is mediated by ADAM10^{211,246}. The resulting soluble L1CAM enhances ERK phosphorylation and cell migration²⁴⁶. In glioma cells, upregulation of ADAM10 at the tumor edges was shown to result in increased soluble L1CAM, interacting with integrin receptors, activation of focal adhesion kinases, and focal complex turnover, all resulting in enhanced migration²¹². ALCAM is also upregulated on many tumors. ALCAM shedding from epithelial ovarian cancer (EOC) cells by ADAM17 interrupts adhesive functions, which is an essential step in EOC cell migration²⁴⁷. In oral squamous cell carcinoma, overexpression of ADAM10 associates with $\alpha v\beta 6$ mediated invasiveness of the tumor cell²¹⁹. In human non-small-cell lung cancer, overexpression of ADAM10 increased the migration and invasion potential of the tumor cells via the activation of the Notch 1 signaling pathway²¹⁴.

Highly metastatic tumors are associated with increased expression of ADAM12²⁴⁸. Overexpression of ADAM12 increases migration and invasion of small cell lung cancer and head and neck squamous cell carcinoma cells^{248,249}. Further, adhesion states for migration and anchorage of melanoma cells are partly regulated by interactions of ADAM12 with $\alpha 9\beta 1$ ²²¹. As already described for ADAM9, ADAM12 exists in a transmembrane and in a soluble form. In particular, ADAM12-S was associated with breast cancer cell invasion *in vitro* and metastasis *in vivo*²²⁰.

ADAM15 promotes the migration and invasion of non-small-cell lung cancer cells by increasing the expression of MMP9 and the conversion of pro-MMP9 to active form²²⁴. In contrast, ADAM15 suppresses vitronectin- and fibronectin-induced cell adhesion and migration in ovarian cancer cells by binding to the $\alpha v\beta 3$ integrin via its RGD domain²²⁶. Interestingly, macrophage- and tumor-derived exosomes containing ADAM15 have a similar effect on cancer cell adhesion and migration via the same mechanism²⁵⁰. Similar

to ADAM15, the proteolytically inactive ADAM23 can function as adhesion molecule by binding to the $\alpha v \beta 3$ integrin and promoting cell-cell interactions and concomitantly modulating metastasis by reducing migration and adhesion to vitronectin²³⁴. Hyaluronic acid, another component of the ECM, is a ligand for the cellular adhesion molecule CD44. ADAM10 and ADAM17 regulate cell-ECM adhesion by shedding CD44, thereby stimulating CD44-mediated migration of adenoma cells through Rac activation^{163,205}.

6.2. Intra- and Extravasation of Cancer Cells

Upon reaching a blood or lymph vessel, invading cancer cells disrupt the endothelial junctions and cross the endothelium into the bloodstream. Surviving circulating cancer cells attach to endothelial cells in a similar manner as leukocytes (as discussed earlier), involving a wide range of ligands including transmembrane chemokines, selectins, integrins, cadherins, CD44, and immunoglobulin superfamily receptors, followed by transmigration.

ADAM8 stimulates endothelial transmigration of triple-negative breast cancer cells via $\beta 1$ -activation *in vitro* and promotes tumor metastasis *in vivo*¹⁹⁹. Eph receptor tyrosine kinases and their membrane-bound ligands, ephrins, are implicated in a variety of cellular responses, including repulsion, attraction, and migration, depending on the cell-type and receptor-ligand binding partners²⁵¹. Eph receptor A1 and ephrin A1 complexes maintain cell-cell adhesion between endothelial cells, however enhancing ADAM12-mediated shedding of ephrin A1 by TGF- $\beta 1$ in primary tumors results in lung hyperpermeability that allows tumor cells to extravasate into the lungs²²². Additionally, knockdown of ADAM15 in a prostate cancer cell line (PC-3) reduced adhesion to and transmigration through a monolayer of endothelial cells *in vitro*. In line, loss of ADAM15 in PC-3 cells injected in SCID mice led to an attenuated bone metastasis²²³. Cleavage of the transmembrane chemokine and adhesion molecule CXCL16 is predominantly mediated by ADAM10²⁵². Increased serum levels of shed CXCL16 have been correlated with high metastasis rate in ovarian cancer cells, which may reflect a prometastatic ADAM10 activity²⁰⁶. Moreover, soluble CXCL16 can mediate the attraction and adhesion of prostate cancer cells via $\alpha v \beta 3$ integrin clustering²⁰⁸. On the other hand, high expression of CXCL16 but low ADAM10 expression, which presumably leads to less CXCL16 shedding and accumulation of transmembrane CXCL16, correlated with longer survival in renal cancer patients. Interestingly, transmembrane CXCL16 in renal cancer cells was found to act proadhesive but antimigratory²⁰⁷.

7. Conclusion and Outlook

In summary, ADAMs 8, 9, 10, 12, 15 and 17 can contribute to cell migration by interfering with distinct steps of cell migration via a number of different effector molecules. This is true for inflammatory cells, various tissue cells, and cancer cells and involves proteolytic cleavage or nonproteolytic interactions of the proteases. Importantly, to promote cell migration, these actions must become efficient at the right time and the right place. For example, shedding of adhesion molecules on leukocytes before the migration process has started will result in less adhesiveness, but once an adherent leukocyte needs to resolve adhesion in order to move to its destination shedding of adhesion molecules may be required. Furthermore, when a tumor cell acquires a motile phenotype, ADAMs can

resolve adhesion contacts to the ECM, thereby allowing dissemination of the tumor, but, on the other hand, when it comes to settlement of this motile cell to form a metastasis shedding of adhesion molecules can also prevent its attachment.

Studies with genetically altered mice indicate which ADAMs may hold a promigratory function in general. Deletion of ADAM10 in leukocytes reduces leukocyte recruitment in a murine model of acute lung inflammation⁸⁶. ADAM10 deficient hepatocellular carcinoma showed less metastasis formation *in vivo*²¹⁷. Transfer experiments also showed a promigratory role of ADAM8 on leukocytes (unpublished data) and on breast and pancreatic cancer cells^{48,199}. Moreover, ADAM9 deficiency in lung cancer cells reduces metastasis in the brain²⁵³. However, deficiency of pathophysiologically important ADAM proteases does not necessarily reduce cell migration in general. For example, deficiency of ADAM17 in leukocytes clearly reduces inflammatory mediator production and reduces the response to bacterial sepsis²⁵⁴, but the absence of this protease does not lead to diminished recruitment of inflammatory cells to inflammatory sites^{86,100,255}.

Thus, inhibition approaches for selected ADAM proteases on leukocytes or cancer cells are warranted. Inhibitor development is most advanced for ADAMs 8, 10, and 17^{32,256}. In endotoxin-induced acute lung inflammation, treatment with a combined ADAM10/17 inhibitor was found to limit leukocyte recruitment²⁵⁷. In a murine model of allergic lung inflammation, ADAM10 inhibition could yield protective effects, which may be partly due to inhibition of leukocyte migration²⁵⁸. Interrupting ErbB receptor transactivation by a combined inhibitor of ADAM10 and ADAM17 reduced non-small-lung-cancer-cell formation, subcutaneous tumor growth, and breast cancer and fibroplasia^{259,260}. Furthermore, also ADAM8 inhibition via a neutralizing antibody or a cyclic peptide inhibitor led to a reduction of allergic lung inflammation, which is associated with a reduction of inflammatory cell infiltration^{78,261}. ADAM8 inhibition could also decrease metastasis of implanted pancreatic tumor cells⁴⁸. ADAM12 inhibitory antibodies or genetically altered tissue inhibitor of metalloproteinases 2 (TIMP2) for specifically blocking ADAM12 or recombinant ADAM12 prodomain still need to be investigated in animal models of cancer cell migration^{262,263}.

The present data indicate that inhibition of selected ADAMs may be of benefit in a situation of severe acute inflammation and/or cancer. However, as summarized in several other reviews, ADAMs also contribute to regenerative processes and additionally, for some less specific metalloproteinase inhibitors, hepatotoxicity has been reported^{32,256}. Therefore, it may be advisable to particularly target ADAMs on immune cells or cancer cells to prevent their undesired migration. The development of bivalent reagents binding to these target cells on the one hand and also specifically blocking ADAM proteases on the other hand may lead to a more specific treatment strategy. This approach may be of particular interest for breast cancers that critically depend on the generation of ErbB-family growth factors via ADAM10 or ADAM17 not only in terms of cell migration and metastasis but also in terms of cell proliferation and tumor growth.

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Chapter 5

Whole body and hematopoietic ADAM8 deficiency
does not influence advanced atherosclerotic lesion
development, despite its association with human
plaque progression

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Abstract

Although A Disintegrin And Metalloproteinase 8 (ADAM8) is not crucial for tissue development and homeostasis, it has been implicated in various inflammatory diseases by regulating processes like immune cell recruitment and activation. ADAM8 expression has been associated with human atherosclerosis development and myocardial infarction, however a causal role of ADAM8 in atherosclerosis has not been investigated thus far. In this study, we examined the expression of ADAM8 in early and progressed human atherosclerotic lesions, in which ADAM8 was significantly upregulated in vulnerable lesions. In addition, ADAM8 was most prominent in the shoulder region of human atherosclerotic lesions, characterized by the abundance of foam cells. In mice, *Adam8* was highly expressed in circulating neutrophils and in macrophages. Moreover, ADAM8 deficient mouse macrophages displayed reduced secretion of inflammatory mediators. Remarkably, however, neither hematopoietic nor whole body ADAM8 deficiency in mice affected atherosclerotic lesion size. Additionally, except for an increase in granulocyte content in plaques of ADAM8 deficient mice, lesion morphology was unaffected. Taken together, whole body and hematopoietic ADAM8 does not contribute to advanced atherosclerotic plaque development, at least in female mice, although its expression might still be valuable as a diagnostic/prognostic biomarker to distinguish between stable and unstable lesions.

Introduction

Atherosclerosis is a lipid-driven chronic inflammatory disease, initiated by endothelial dysfunction, resulting in the subendothelial accumulation and modification of circulating lipoprotein particles, together with the recruitment of leukocytes within the vessel wall. These modified lipoproteins are internalized by macrophages, forming foam cells which eventually become apoptotic. Progressed atherosclerotic lesions are characterized by the formation of a fibrous cap and a lipid rich necrotic core ¹. These lesions may, upon rupture, lead to local thrombosis, the major cause of clinical events such as myocardial infarction and stroke ².

A Disintegrin And Metalloproteinases (ADAMs) are a family of transmembrane proteases which play a role in modulating inflammatory responses ³. Their role in cardiovascular diseases (CVD)/ atherosclerosis is emerging as evidenced by several recent publications showing that ADAM10 modulates atherosclerotic plaque composition ⁴, while ADAM15 contributes to lesion development ⁵ and ADAM17 provides with atherosclerosis resistance ^{6,7}. Among several members of this family, ADAM8 exhibits sheddase activity, enabling cleavage of atherosclerosis related cell surface proteins, including the inflammatory molecules L-selectin, P-selectin glycoprotein ligand-1 (PSGL-1), tumor necrosis factor (TNF), TNF receptor 1 and vascular cell adhesion molecule 1 ^{8,9}. ADAM8 is highly expressed in most cells of hematopoietic origin, and in the brain, bone, lung and thymus ¹⁰⁻¹⁵. Despite being widely expressed, mice deficient in ADAM8 have a normal development with no overt phenotype ¹⁵. With respect to pathologies, ADAM8 levels increase in cancer and inflammatory diseases of the lung, central nervous system, bone and joints, and its expression positively correlates with disease severity ^{11,16-18}.

ADAM8 expression was also upregulated in human atherosclerotic plaques compared to non-atherosclerotic control vessels ¹⁹. Moreover, this study showed specific ADAM8 polymorphisms (rs2995300C and rs2275725A) to be associated with atherosclerosis development and myocardial infarction in two independent human cohorts. However, it is unclear whether ADAM8 is also causally involved in atherosclerosis development. In this study, we investigated whether ADAM8 plays a role in the development and progression of atherosclerotic lesions. Although we show that ADAM8 expression is associated with lesion progression in human disease, genetic ablation of ADAM8 both in the hematopoietic compartment as well as at whole body level did not affect advanced atherosclerosis development in female mice.

Results

ADAM8 expression increases with atherosclerotic plaque progression in humans and is mainly associated with foam cell-rich regions

Reanalysis of a previous microarray study²⁰ (GSE7074) from our laboratory comparing human atherosclerotic plaque macrophages and resident macrophages derived from liver, lung and spleen revealed ADAM8 as one of the highest upregulated genes in plaque macrophages (**fig. 1a**), which prompted us to investigate ADAM8 gene expression in different stages of human atherosclerotic plaque development. Tissue lysates from early, stable and unstable human lesions showed significantly increased ADAM8 mRNA expression in unstable lesions (**fig. 1b**).

Immunohistochemical staining of ADAM8 in human carotid atherosclerotic plaques confirmed the expression of ADAM8 in lesions at the protein level. ADAM8 expression was not only found in leukocytes (as previously reported¹³), but also in luminal and microvascular endothelial cells and, potentially, vascular smooth muscle cells (**suppl. fig. 1a and b**). ADAM8 localized intensely to the shoulder regions of the plaque (**fig. 1c**), an area mainly composed of inflammatory cells²¹.

Figure 1. ADAM8 expression in human atherosclerotic lesions.

(**a**) Microarray expression profiling of carotid atherosclerotic plaque macrophages and lung, liver and spleen macrophages ($n = 4$ patients per tissue, nonparametric Mann-Whitney U test). (**b**) Relative expression of ADAM8 mRNA in early, advanced stable and unstable human plaques. Values have been corrected for GAPDH expression ($n = 5-6$) and ADAM8 expression levels in early plaques were normalized to 1 (one way analysis of variance followed by Dunn's multiple comparison test). (**c, d**) Immunohistochemical stainings for ADAM8 in human atherosclerotic lesions. (**c**) Representative images of a shoulder region (scale bar, 400 μ m), a region rich in foamy macrophages (scale bar, 50 μ m) and a negative control (**d**, scale bar, 200 μ m) are shown.

Adam8 is mainly expressed in neutrophils and macrophages and modulates secretion of inflammatory mediators

Leukocytes play a pivotal role in both human and murine atherosclerosis development²². In homeostatic conditions, expression of ADAM8 is mainly restricted to cells of the immune system⁸. We therefore sought to examine its expression in various leukocyte subsets isolated from wildtype C57Bl/6 mice. Interestingly, *Adam8* mRNA is mainly expressed in circulating neutrophils and, to a lower extent, in bone marrow-derived macrophages (BMDMs; **fig. 2a**). In contrast, expression of *Adam8* mRNA in monocytes and B- and T-lymphocytes was barely detectable. ADAM8 expression was reported to increase under pathological conditions^{11,16-18}. Since macrophages are the main inflammatory cell type in mouse atherosclerotic plaques where they will be exposed to (modified) lipoproteins²³, we investigated *Adam8* expression in BMDMs exposed to different types of lipoproteins. Interestingly, while *Adam8* mRNA levels were not affected by very low-density lipoproteins (VLDLs) or low-density lipoproteins (LDL), oxidized LDL increased ADAM8 mRNA (**fig. 2b**) and protein (**fig. 2c**) expression in BMDMs. This is in line with the pronounced ADAM8 expression in foamy macrophages of human atherosclerotic plaques (**fig. 1c**). Since ADAMs play a crucial role in modulating inflammatory responses, we examined the role of ADAM8 in LPS-induced cytokine production by BMDMs. Interestingly, ADAM8 deficiency resulted in significantly reduced TNF α , interleukin (IL)-10 and IL-12 as well as nitric oxide (NO) secretion, both when BMDMs were pre-exposed to oxLDL followed by LPS or LPS alone (**fig. 2d**).

Figure 2. *Adam8* expression in leukocyte subsets and ADAM8 deficient BMDMs show reduced inflammatory response.

(a) Relative *Adam8* mRNA expression in sorted murine C57Bl/6 blood B-lymphocytes, T-lymphocytes, monocytes, neutrophils, resident peritoneal macrophages and BMDMs ($n = 4$ mice). Fold changes of *Adam8/Gapdh* expression is shown. (b) *Adam8* expression in wildtype C57Bl/6 BMDMs exposed for 24h to 0.25 μ g/ml VLDL, LDL or oxLDL ($n = 2$ per group). (c) Western blot analysis of BMDMs stimulated with PBS or 0.25 μ g/ml oxLDL ($n = 3$ per group). (d) Cytokine or nitric oxide (NO) secretion by *Adam8*^{+/+} and *Adam8*^{-/-} BMDMs that were pre-treated with PBS or 0.25 μ g/ml oxLDL for 24 hours, followed by a 6 hours (TNF α and IL-10) or 24 hours (IL-12 and NO) incubation with PBS or 10 ng/ml LPS ($n = 3$ mice per group, nonparametric Mann-Whitney U test).

Hematopoietic ADAM8 deficiency does not affect size or composition of advanced atherosclerotic lesions

Considering its expression in hematopoietic cells and its role in modulating macrophage inflammatory responses, we investigated the effect of hematopoietic deficiency of ADAM8 on lesion development by reconstituting lethally irradiated female low-density lipoprotein deficient (*Ldlr*^{-/-}) mice with bone marrow from either ADAM8 deficient (*Adam8*^{-/-}→*Ldlr*^{-/-}) mice or wildtype (*Adam8*^{+/+}→*Ldlr*^{-/-}) littermate controls. The efficiency of the bone marrow transplantation (BMT) was 96%±0.1. After reconstitution, mice were fed a western type diet (WTD) for 10 weeks after which lesions development was assessed. No differences between genotypes were found in body weight (**suppl. fig. 2a**) or plasma cholesterol before and after WTD (**fig. 3a**). Although at baseline triglyceride levels in *Adam8*^{-/-}→*Ldlr*^{-/-} mice were slightly decreased compared to *Adam8*^{+/+}→*Ldlr*^{-/-} mice, no differences were observed upon WTD (**fig. 3b**). Furthermore, after recovery from the BMT, circulating leukocyte counts were analyzed by flow cytometry. Both at baseline (before WTD) and after 10 weeks of WTD the total number of CD45⁺ leukocytes in blood was significantly reduced in hematopoietic ADAM8 deficient mice compared to wildtype controls (**suppl. fig. 2b and c**). ADAM8 deficiency associated leukopenia affected almost all major leukocyte (sub)populations.

After 10 weeks of WTD feeding, atherosclerotic lesion area was not affected by hematopoietic ADAM8 deficiency, both in the aortic root (**fig. 3c**) and brachiocephalic artery (**suppl. fig. 2d**). Furthermore, there was no change in necrotic core content between both groups in the aortic root (**fig. 3d**). Routine pathological examination for atherosclerotic plaque progression (**fig. 3e**) as well as (immuno)histochemical stainings for macrophage (**fig. 3e**), neutrophil (**fig. 3f**) and collagen content (**fig. 3g**) showed no significant differences between *Adam8*^{-/-}→*Ldlr*^{-/-} and *Adam8*^{+/+}→*Ldlr*^{-/-} mice. Moreover, mRNA levels of *Arg1* and *Nos2*, were unchanged between both genotypes (**suppl. fig. 2e**), suggesting no difference in macrophage polarization. Moreover, we did not observe a compensatory upregulation of the related metalloprotease *Adam17*. Altogether, these data show that, despite reduced amounts of circulating leukocytes, hematopoietic ADAM8 deficiency does not influence advanced atherosclerotic lesion development or composition.

Figure 3. Hematopoietic ADAM8 deficiency in $Ldlr^{-/-}$ mice does not affect size or morphology of advanced atherosclerotic lesions.

(a, b) Plasma cholesterol (a) and triglyceride (b) levels after 0, 5 and 10 weeks of WTD feeding in female $Ldlr^{-/-}$ chimeras with ($Adam8^{+/+} \rightarrow Ldlr^{-/-}$) or without ($Adam8^{-/-} \rightarrow Ldlr^{-/-}$) hematopoietic ADAM8 deficiency ($n = 20$ mice per genotype, parametric Student's t -test). (c, d) Quantification of the aortic root lesion area (c, non-parametric Mann-Whitney U test) and necrotic core area (d, parametric Student's t -test) of $Adam8^{+/+} \rightarrow Ldlr^{-/-}$ and $Adam8^{-/-} \rightarrow Ldlr^{-/-}$ mice ($n = 18$ mice per genotype) after 10 weeks of WTD. (e) Plaque progression stage ($n = 51$ aortic root atherosclerotic lesions per genotype) was scored (Fisher's exact test). (f-h) Representative examples of (immuno)histochemical stainings and quantifications for MOMA-2⁺ macrophages (f, $n = 18/16$ mice, parametric Student's t -test, scale bar, 200 μm), NIMP⁺ neutrophils (g, $n = 18/16$ mice, non-parametric Mann-Whitney U test, scale bar, 50 μm) and Sirius Red stained collagen (h, $n = 18/16$ mice, parametric Student's t -test, scale bar, 200 μm) in the aortic root after 10 weeks of WTD feeding.

Advanced atherosclerotic lesions are not affected by whole-body ADAM8 deficiency

Most ADAM family members are expressed as transmembrane proteases. ADAM8, however, also exists in a soluble form comprising the, functional, metalloproteinase domain only^{24,25}. Although significantly lower compared to wildtype controls, hematopoietic ADAM8 deficient mice still have considerable plasma levels of soluble ADAM8 (**suppl. fig. 2f**) most likely originating from non-hematopoietic cells which may have compensated for the hematopoietic deficiency.

To exclude this possibility, we rendered female whole-body *Adam8*^{-/-} and wildtype littermate controls prone to atherosclerosis by using a recently developed proprotein convertase subtilisin/kexin type 9 (PCSK9) overexpressing adeno-associated viral vector in combination with WTD feeding²⁶. Indeed, PCSK9 overexpression raised both plasma cholesterol and triglyceride levels upon WTD feeding to a similar extent as in *Ldlr*^{-/-} mice, but both were not found different between *Adam8*^{-/-} and wildtype mice (**fig. 4a and b**), despite a lower body weight in *Adam8*^{-/-} mice after 10 weeks of WTD (**suppl. fig. 3a**). Additionally, blood leukocyte counts were measured using flow cytometry before animals were subjected to WTD feeding and at sacrifice (**suppl. fig. 3b and c**). In contrast to hematopoietic ADAM8 deficient mice, no leukopenia was observed and whole-body ADAM8 deficiency even resulted in slightly increased T cell and NK cell counts under hyperlipidemic conditions.

Surprisingly, even whole-body ADAM8 deficiency did not affect atherosclerotic lesion size in the brachiocephalic artery (**suppl. fig. 3d**) and aortic root (**fig. 4c**). Similar to hematopoietic ADAM8 deficient mice, necrotic core content (**fig. 4d**), plaque stage (**fig. 4e**), as well as (immuno)histochemical staining of macrophage (**fig. 4f**) and collagen content (**fig. 4h**) showed no difference in whole body *Adam8* knockout mice compared to wildtype controls. Moreover, *Arg1*, *Nos2* and *Adam17* mRNA expression was similar between both genotypes (**suppl. fig. 3e**). However, the amount of granulocytes was significantly increased in ADAM8 deficient mice compared to wildtype mice (**fig. 4g**). Collectively, these data show that both hematopoietic as well as whole-body ADAM8 deficiency does not affect advanced atherogenesis in mice.

Figure 4. Whole body ADAM8 deficiency has no effect on advanced plaque size or morphology.

(a, b) Plasma cholesterol (a) and triglyceride (b) levels of female whole-body wildtype (*Adam8*^{+/+}) and *Adam8*^{-/-} mice rendered hyperlipidemic by AAV8-PCSK9 gene transfer and subsequent WTD feeding, at start of WTD diet (0) and after 5 and 10 weeks (*n* = 16/14 mice, parametric Student's *t*-test). (c, d) Quantification of the aortic root lesion area (c, *n* = 14/13 mice, nonparametric Mann-Whitney *U* test) and necrotic core area (d, *n* = 14/13 mice, nonparametric Mann-Whitney *U* test) of whole body *Adam8*^{+/+} and *Adam8*^{-/-} mice after 10 weeks of WTD feeding. (e) Plaque stage classification (*n* = 42/39 atherosclerotic lesions in the aortic root) was scored (Fisher's exact test). (f-h) Representative examples of (immuno)histochemical stainings for MAC-3⁺ macrophages (f, *n* = 14/13 mice, parametric Student's *t*-test scale bar, 200 μm), Ly6G⁺ granulocytes (g, *n* = 14/13 mice, nonparametric Mann-Whitney *U* test scale bar, 50 μm) and Sirius Red stained collagen (h, *n* = 14/13 mice, nonparametric Mann-Whitney *U* test scale bar, 200 μm) with quantification.

Discussion

This is the first study that investigated the role of ADAM8 in atherosclerosis development. Although, we clearly showed that ADAM8 expression is increased in unstable human atherosclerotic lesion, we did not observe any effects of hematopoietic nor whole-body ADAM8 on advanced atherosclerosis development in mice.

Originally identified in a human macrophage cell line ²⁷, ADAM8 is mainly expressed in most cells of hematopoietic origin, both under physiological as well as inflammatory conditions ^{13,28}. Here, we show that in mice, under physiological conditions, *Adam8* indeed is highly expressed in circulating neutrophils and to a lower extent in bone marrow derived macrophages. In contrast, *Adam8* is hardly expressed by monocytes and lymphocytes, in agreement with open access mouse leukocyte expression databases (immgen.org). Similarly, in humans ADAM8 was also seen to be expressed by neutrophils ²⁵ and monocytes ^{13,29}, while T lymphocytes lack ADAM8 expression ¹³. Interestingly, however, human B lymphocytes do express ADAM8, albeit to a lower extent compared to human monocytes ¹³. These findings suggest species differences in hematopoietic ADAM8 expression, at least in T and B lymphocytes. Next to neutrophils, macrophages express higher levels of *Adam8* than monocytes and lymphocytes under physiological conditions. Interestingly, peritoneal macrophages, which are a subset of resident macrophages, express similar low *Adam8* levels as monocytes. The difference in *Adam8* expression between freshly differentiated macrophages from bone marrow cells and resident macrophages might be due to the difference in their local microenvironment which initiate different transcriptional programs ³⁰ or their mode of differentiation ³¹.

The atherosclerotic plaque harbors multiple environmental cues which dynamically regulate the expression profiles of macrophages ³². Previously, it has been shown that ADAM8 gene and protein expression increase upon atherosclerotic lesion development in humans, and that its expression co-localized with plaque smooth muscle cells and macrophages ³³. In this study, we show that ADAM8 is especially abundant in the plaque shoulder regions, which are rich in leukocytes, especially (foamy) macrophages. Furthermore, oxLDL, which is abundantly present in atherosclerotic lesions and can modify macrophage phenotype and function ²³, was seen to upregulate ADAM8 expression, unlike LDL and VLDL. Interestingly, ADAM8 deficient mouse macrophages have an attenuated secretion of inflammatory mediators, including TNF, a proposed substrate of ADAM8 ³⁴. Despite ADAM17 being the main TNF sheddase both *in vitro* and *in vivo* ^{35,36}, ADAM8 deficiency has a significant contribution to TNF release *in vitro*. We found both pro- and anti-inflammatory cytokines to be reduced, i.e. no clear M1 or M2 profile, in line with unchanged *Nos2* and *Arg-1* expression in plaques *in vivo*.

Remarkably, despite the absence of any effects of hematopoietic ADAM8 deficiency on atherosclerosis development, these bone marrow transplanted mice displayed a slight decrease in triglyceride levels at baseline and mild leukopenia both under normo- and hyperlipidemic conditions. Although only evident during recovery after bone marrow transplantation, the potential role of hematopoietic ADAM8 deficiency in triglyceride metabolism remains to be determined. Regarding the observed leukopenia, ADAM8

has previously been shown to regulate the surface expression of the adhesion molecules L-selectin and PSGL-1 through shedding ^{25,28}. Interestingly, ligation of PSGL-1 on hematopoietic progenitor cells to P-selectin results in suppression of hematopoiesis ³⁷. Additionally, L-selectin may function as a retention cue for granulocytes to remain in the bone marrow ^{38,39}. Although ADAM8 might indirectly affect both processes, via these two substrates, the fact that whole-body ADAM8 deficient mice failed to display leukopenia does not plead for this notion. Another possibility for this discrepancy might lie in a role for ADAM8 in the homing of progenitor cells towards the bone marrow niche after BMT. Furthermore, ADAM8-mediated shedding of the adhesion molecules L-selectin and PSGL-1 may also control neutrophil recruitment ^{25,28}. ADAM8 deficiency results in increased surface expression of both molecules, which will lead to increased tethering of neutrophils to endothelial cells ⁴⁰. In line, atherosclerotic lesions of whole-body ADAM8 deficient mice contained increased amounts of granulocytes compared to their respective controls. In contrast, hematopoietic ADAM8 deficiency did not result in an accumulation of neutrophils in atherosclerotic lesions, albeit this might be due to the presence of sufficient levels of plasma sADAM8, which also has the capacity to cleave transmembrane proteins, like L-selectin. Indeed, transgenic mice that overexpressed the soluble form of ADAM8 display a reduction in surface expression of L-selectin and concomitantly a decrease in leukocyte accumulation in the peritoneal cavity after receiving an inflammatory stimulus ⁴¹. Similarly, overexpression of ADAM8 might also attenuate leukocyte recruitment towards atherosclerotic lesions, and thus reduce atherosclerotic burden. On the other hand, however, overexpression of ADAM8 may also increase the inflammatory response of macrophages and other cell types that have the capacity to secrete TNF α ³⁴. Therefore, ADAM8 affects processes that inhibit or exacerbate atherosclerosis development, which could explain why we did not observe any change in plaque size or morphology in female ADAM8 deficient mice. Note, that we cannot exclude the possibility that ADAM8 differentially affects atherosclerosis development in males.

In addition to the potential dual effects of ADAM8 in atherosclerosis, other ADAMs with a similar substrate profile as ADAM8, such as ADAM17 ⁴², might at least partly compensate for the loss of ADAM8. ADAM17 expression was unchanged between ADAM8 wildtype and knockout mice in both atherosclerosis models, suggesting there is no overcompensation for the loss of ADAM8 deficiency although this does not rule out any functional compensation.

Furthermore, neutrophils are involved in the process of atherosclerosis, primarily during the initiation of lesion development ⁴³. Therefore, it cannot be excluded that ADAM8 might have an effect at earlier developmental stages, since all atherosclerotic lesions in this study were classified as moderate to advanced.

We and others ^{33,44} have shown that ADAM8 expression is increased in advanced human atherosclerotic lesions. Next to its expression in macrophages, especially in foam cells located at the shoulder region of the atherosclerotic lesion, ADAM8 was also expressed in plaque stromal cells, including endothelial cells and, potentially, smooth muscle cells, though the role of ADAM8 in these vascular cell types is less defined. Risk allele carriers have increased serum levels of soluble ADAM8 and an increased risk of myocardial infarction ¹⁹, which shows that ADAM8 may still have clinical potential.

In conclusion, while ADAM8 affects inflammatory responses *in vitro*, our data argue against a critical role for both hematopoietic and whole-body ADAM8 in atherosclerosis development in female mice, at least in advanced stages of the disease. However, ADAM8 may still be useful as a diagnostic/prognostic biomarker to distinguish between stable and unstable atherosclerotic lesions in humans, though further research is needed.

Materials and Methods

Microarray and quantitative PCR analysis of human tissues. Human carotid artery plaque tissue⁴⁵ and nonatherosclerotic (lung, liver, spleen) tissues²⁰ were obtained by endarterectomy or autopsies, respectively, as previously described. Collection, storage and use of tissue in the Maastricht Pathology Tissue Collection (MPTC) and patient data confidentiality were performed after informed consent and in agreement with the 'Code for Proper Secondary Use of Human Tissue in the Netherlands' and in accordance with the guidelines of, and approved by the medical and ethical committee of Maastricht University Medical Centre, Maastricht, The Netherlands. Sample processing, macrophage isolation and microarray hybridization and analysis of the differences between carotid plaque, lung, liver and spleen macrophages was performed as previously described (Gene Expression Omnibus database accession number GSE7074)²⁰. Carotid lesion segments for quantitative PCR analysis were snap-frozen and subsequently RNA was isolated using the guanidine isothiocyanate/CsCl method as previously described⁴⁵. RNA was further purified and concentrated using RNeasy mini columns (Qiagen). Total RNA was normalized and reverse transcribed using iScript (Biorad). Quantitative PCR (qPCR) was performed using 10 ng cDNA, 300 nM of each primer, and SensiMix (Quantace-Bioline). All gene expression levels were corrected for cyclophilin A and β -actin as housekeeping genes.

Immunohistochemistry of human tissues. Human carotid endarterectomy segments were fixed in paraformaldehyde and paraffin embedded. Sections were incubated with a primary antibody against human ADAM8 (AF1031, R&D systems), followed by detection with a biotin-labelled rabbit anti-goat antibody (E0466, Dako) and Vector Red ABC kit (Vector Labs, CA, USA). Atherosclerotic lesions were classified as introduced by Virmani *et al.*⁴⁶. Pathological intimal thickening or xanthomata were defined as 'early', thick fibrous cap atheroma 'stable' and lesions with a thrombus or intraplaque hemorrhage 'unstable' lesions.

Animals. Mouse experiments were approved by the Animal Ethics Committee of Maastricht University, the Netherlands (permit number 2012-065), and were performed in compliance with the Dutch government guidelines. Female *Ldlr*^{-/-} mice were obtained from an in-house breeding colony, originally derived from Charles River (Wilmington, MA, USA). Female *Adam8* knockout (*Adam8*^{-/-}) and wildtype (*Adam8*^{+/+}) littermate control mice on a C57Bl/6 background were previously described and generously provided by Dr. J. Bartsch¹⁵.

Peritoneal macrophage isolation. Resident peritoneal macrophages were obtained by flushing the peritoneal cavity with ice-cold phosphate buffered saline (PBS) followed by culturing in RPMI 1640 Glutamax containing 10% (vol/vol) heat inactivated fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100ug/ml), and L-glutamine 2mM (all GIBCO Invitrogen, Breda, the Netherlands). After overnight attachment, cells were washed three times with RPMI 1640 Glutamax to remove non-adherent cells. Adherent cells were dissolved in TRIzol reagent (Life Technologies) and stored at -20°C until

further use.

Bone marrow-derived macrophage isolation and culture. Bone marrow cells were isolated from femurs and tibiae of either *Adam8*^{-/-} or wildtype littermate controls. Cells were cultured in RPMI 1640 Glutamax (GIBCO Invitrogen, Breda, the Netherlands) with 10% (vol/vol) heat-inactivated FCS (Bodinco B.V. Alkmaar, the Netherlands), penicillin (100 U/ml) and streptomycin (100 µg/ml) supplemented with 15% L929-conditioned medium (LCM) for 8 days to generate bone-marrow derived macrophages (BMDMs). BMDMs were seeded at 0.35 x 10⁶ cells per well in 24 wells plates and incubated 6-24 hours with 10 ng/ml LPS (*E. Coli* 055:B5, Sigma). Alternatively, BMDMs were incubated for 24 hours with 25 µg/ml very-low density lipoprotein (VLDL), low density lipoprotein (LDL) or oxidized LDL (oxLDL). VLDL and LDL were isolated from human serum ⁴⁷ and LDL oxidatively modified by CuSO₄ as previously described ⁴⁸. Supernatants were collected and stored at -20°C until further use.

Fluorescence-activated cell sorting. Leukocyte subsets were isolated from blood collected from wildtype C57Bl/6 mice using a FACS Aria (BD Biosciences). Erythrocytes were removed by incubation with erylysis buffer (155 mM NH₄Cl and 10 mM KHCO₃). Leukocytes were defined as CD45⁺ (Biolegend), T-lymphocytes as CD45⁺ CD3⁺ (eBioscience) NK1.1⁻ (BD), B-lymphocytes as CD45⁺ CD3⁻ NK1.1⁻ B220⁺ (BD), granulocytes as CD45⁺ CD3⁻ NK1.1⁻ B220⁻ CD11b⁺ (BD) Ly6G⁺ (BD) and monocytes as CD45⁺ CD3⁻ NK1.1⁻ B220⁻ CD11b⁺ Ly6G⁻.

Quantitative PCR in murine cells. RNA from FACS sorted cells and murine aortic arches was isolated using TRIzol (Life Technologies) or RNeasy mini kit (Qiagen), respectively, according to the manufacturer's instructions. RNA (500 ng) was reverse transcribed using the iScript cDNA Synthesis Kit (Biorad). Quantitative PCR was performed using 10 ng cDNA, 300 nmol/L of each primer and Sensimix SYBR Green (Biorad) in a total volume of 12 µL. All gene expression levels were expressed relative to *Gapdh* as housekeeping genes. Primer sequences are available upon request.

ELISA. TNF, IL-10 (after 6h LPS) and IL-12p40 (after 24h LPS) levels in BMDM-derived conditioned medium were measured with ELISA kits (Invitrogen) according to the manufacturer's instructions. Plasma soluble ADAM8 was measured using a commercial ELISA kit (Hoelzel Diagnostica, Cologne, Germany) according the manufacturer's instructions. Analysis was performed using a micro-plate reader (Biorad).

NO assay. NO₂⁻ concentrations were determined in LPS-induced BMDM-derived conditioned medium using Griess reagent (2.5% H₃PO₄, 1% sulphanilamide, 0.1% naphthalene diamine dihydrochloride) and measured at 540 nm (benchmark microplate reader, Biorad).

Western Blot analysis. After a 24 hours incubation with PBS or 25 µg/ml oxLDL, BMDMs were treated lysis buffer (150 mM NaCl, 50 mM Tris, 10 mM EDTA, 1% NP-40, 8.7% glycerol, 0.1% SDS) supplemented with 1x Complete Inhibitor (Roche).

After centrifugation at 16.000 x g for 5 min, supernatants were collected and protein concentration was determined using bicinchoninic acid (BCA) assay (ThermoFisher). Supernatants were investigated by reducing SDS-PAGE as previously described ⁴. Proteins were transferred onto nitrocellulose membrane (GE Healthcare Life Sciences). Membranes were blocked with 5% (w/v) non-fat dry milk in Tris buffered saline with 0.1% Tween (TBS-T) for 1 hour and probed with primary antibody against murine ADAM8 (Abcam) or β -actin (Abcam) overnight, followed by appropriate horseradish peroxidase conjugated secondary antibodies. Chemiluminescence was detected using ImageQuant LAS 4000 mini (GE Healthcare Life Sciences). Images of full-length blots are shown in **suppl. figure 4**.

Bone marrow transplantation and atherosclerotic lesion analyses. Female wildtype and *Adam8*^{-/-} mice (aged 10 to 12 weeks) were used as donor mice ($n = 4$ per group). Female *Ldlr*^{-/-} recipient mice (10 weeks old, $n = 18$ per group), backcrossed onto a C57Bl/6 background for >10 generations, were obtained from in-house breeding. Bone marrow transplantations (BMT) were performed as described elsewhere ⁴⁵. Briefly, *Ldlr*^{-/-} mice were lethally irradiated with 6 Gy a day before and on the day of transplantation. Mice were transplanted with 5×10^6 bone marrow cells isolated from wildtype or *Adam8*^{-/-} mice. After a recovery period of 5 weeks after transplantation, mice were given a western type diet (WTD) containing 0.25% cholesterol (Special Diets Services, Witham, Essex, UK). Before WTD feeding, mice were fasted for 4 hours, after which blood samples were drawn from the tail vein for analyses of plasma lipids, chimerism, soluble ADAM8 and leukocytes. Additional blood analyses were performed at 5 and 10 weeks of WTD, after 4 hours fasting. After 10 weeks of WTD feeding, mice were anesthetized, euthanized and perfused with PBS containing nitroprusside (0.1 mg/ml, Sigma).

Mouse hearts were dissected and snap-frozen in optimum cutting temperature medium. Serial cryosections (7 μ m) of the aortic root and brachiocephalic artery were fixed in acetone and stained with toluidine blue for morphometric analysis of plaque and necrotic core area (defined as acellular regions). Total plaque area per mouse was defined as the average plaque area of four consecutive toluidine blue-stained sections at 42 μ m intervals. Plaque stages were determined as previously described ⁴⁹, with slight modifications. Plaques were classified as early (foam cell rich, but lacking a necrotic core), moderately advanced (containing a fibrotic cap and often a necrotic core, but no medial macrophage infiltration) and advanced lesions, typified by medial macrophage infiltrates, elastic lamina degradation and more pronounced necrosis and fibrosis. MOMA-2 (an antibody recognizing monocytes/macrophages; gift from G. Kraal), NIMP-1 (neutrophil specific antibody directed against Ly6G; gift from P. Heeringa) and Sirius Red (Sigma) staining was used for the detection of monocytes/macrophages, neutrophils and collagen, respectively. Pictures were taken using a Leica DM3000 light microscope (Leica Microsystems, Wetzlar, Germany) and sections were analyzed in a blinded manner using Adobe Photoshop CS6 software (Adobe, San Jose, US).

Atherosclerotic lesion analysis in whole-body ADAM8 deficient mice. Female wildtype and *Adam8*^{-/-} mice (aged 10 to 12 weeks; $n = 16$ and 14, respectively) were rendered prone to atherosclerosis by a single intravenous injection of adeno-associated virus serotype

8 containing D377Y-murine PCSK9 (AAV-PCSK9; 1×10^{11} vector genomes per mouse; as described previously ²⁶), followed by WTD feeding (0.25% cholesterol; Special Diets Services, Witham, Essex, UK). Blood was collected from the tail vein for analyses of plasma lipids at baseline (before WTD) and after 5 and 10 weeks of WTD feeding after 4 hours fasting. After 10 weeks of WTD feeding, mice were anesthetized, euthanized and perfused with PBS containing nitroprusside (0.1 mg/ml, Sigma). Mouse hearts were excised and fixed overnight in 1% paraformaldehyde. Serial paraffin sections of the aortic root and brachiocephalic artery were cut (4 μ m) and stained with hematoxylin and eosin (H&E, Sigma) for morphometric analysis of plaque and necrotic core area (defined as acellular regions), and plaque staging, as described in the BMT section. Total plaque area per mouse was defined as the average plaque area of five consecutive H&E-stained sections at 20 μ m intervals. Atherosclerotic lesions were further characterized for macrophage (MAC3, BD), granulocyte (Ly6G, Becton & Dickenson) and collagen (Sirius Red) content. Similar as to the BMT setup, pictures were taken using a Leica DM3000 light microscope and sections were analyzed in a blinded manner using Adobe Photoshop CS6 software.

Flow cytometry analyses and blood lipid analyses. Absolute circulating leukocyte subset numbers were determined by flow cytometry calibrated using Trucount Beads (BD, New Jersey, US). Blood was collected at the start ($t = 0$) and after 10 weeks of WTD. Erythrocytes were removed by incubation with erylysis buffer (155 mM NH_4Cl and 10 mM KHCO_3). Leukocytes were defined as CD45^+ (Biolegend), T-lymphocytes as $\text{CD45}^+ \text{CD3}^+$ (eBioscience) NK1.1^- (BD), NK cells as $\text{CD45}^+ \text{CD3}^- \text{NK1.1}^+$, B-lymphocytes as $\text{CD45}^+ \text{CD3}^- \text{NK1.1}^- \text{B220}^+$ (BD) granulocytes as $\text{CD45}^+ \text{CD3}^- \text{NK1.1}^- \text{B220}^- \text{CD11b}^+$ (BD) Ly6G^+ (BD) and monocytes as $\text{CD45}^+ \text{CD3}^- \text{NK1.1}^- \text{B220}^- \text{CD11b}^+ \text{Ly6G}^-$. Monocyte subsets were further distinguished based on the degree of Ly6C expression. Data were acquired using a FACS Canto II (BD Bioscience) and analyzed with FACSdiva software (BD Bioscience). Blood was collected at the start ($t = 0$) and after 5 and 10 weeks of WTD. Plasma was separated by centrifugation (2100 x g, 10 minutes, 4°C), and stored at -80°C until further use. Plasma cholesterol and triglycerides were determined using standard enzymatic kits (Cholesterol FS'10; Triglycerides FS 5' Ecoline; Diagnostic Systems GmbH, Holzheim, Germany) according to the manufacturer's instructions.

Statistics. Data are presented as mean $\pm$ SEM. All statistical analyses were performed using the Prism software (GraphPad Software version 5, San Diego, CA, USA). Differences between two groups or more were evaluated for statistical significance with the appropriate Student's *t* test, Mann-Whitney *U* test, Fisher's exact test or one-way analysis of variance followed by Dunn's multiple comparisons test, respectively. *P* values of less than 0.05 were considered significant.

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Supplemental figures

Supplemental figure 1. ADAM8 is expressed in human atherosclerotic lesions.

(a-i) Immunohistochemical staining of ADAM8 in a human atherosclerotic lesion (scale bar, 400 µm). Representative images of (a-ii) luminal endothelial cells (scale bar, 50 µm) and (a-iii) smooth muscle cells (scale bar, 50 µm; indicated by black arrows) are shown. (b) Representative immunohistochemical staining of ADAM8 in (b-i) human atherosclerotic lesion (scale bar, 400 µm) and (b-ii) microvessels is shown (scale bar, 100 µm).

Supplemental figure 2. Hematopoietic ADAM8 deficiency reduces total blood leukocyte levels, but does not affect lesion size in the brachiocephalic artery.

(a) body weight over the course of 10 weeks of western type diet (WTD) in *Ldlr*^{-/-} chimeras with (*Adam8*^{-/-}→*Ldlr*^{-/-}) or without (*Adam8*^{+/-}→*Ldlr*^{-/-}) hematopoietic ADAM8 deficiency (*n* = 20 mice per group, parametric Student's *t* test). (b) Blood counts of several leukocyte subsets at baseline (T = 0; *n* = 10 mice per group) and after 10 weeks of WTD feeding (T = 10; *n* = 10 mice per group, parametric Student's *t* test). (c) Ly6C^{low} and Ly6C^{high} monocyte subsets in blood at baseline and after 10 weeks of WTD (*n* = 10 mice per group, parametric Student's *t* test). (d) Quantification of the brachiocephalic artery lesion area in *Adam8*^{-/-}→*Ldlr*^{-/-} and *Adam8*^{+/-}→*Ldlr*^{-/-} mice (*n* = 8/9 mice) after 10 weeks of WTD (nonparametric Mann-Whitney *U* test). (e) mRNA expression levels in aortic arches of *Adam8*^{-/-}→*Ldlr*^{-/-} and *Adam8*^{+/-}→*Ldlr*^{-/-} (*n* = 5/8 mice) after 10 weeks of WTD (nonparametric Mann-Whitney *U* test). (f) plasma soluble ADAM8 protein 10 weeks of WTD feeding in *Adam8*^{-/-}→*Ldlr*^{-/-} and *Adam8*^{+/-}→*Ldlr*^{-/-} mice (*n* = 19/20 mice, nonparametric Mann-Whitney *U* test).

Supplemental figure 3. Whole-body ADAM8 deficient mice display increased T- and NK-cells after 10 weeks of WTD, but no change in brachiocephalic artery lesion size.

(a) body weight over the course of 10 weeks of western type diet (WTD) in ADAM8 deficient ($Adam8^{-/-}$) and wildtype ($Adam8^{+/+}$) mice ($n = 16/14$ mice, nonparametric Mann-Whitney U test). (b) Blood counts of several leukocyte subsets at baseline (T = 0; $n = 10$ mice per group) and after 10 weeks of WTD (T = 10; $n = 10$ mice per group, parametric Student's t test). (c) Ly6C^{low} and Ly6C^{high} monocyte subsets in blood at baseline and after 10 weeks of WTD ($n = 10$ mice per group, parametric Student's t test). (d) Quantification of the brachiocephalic artery root lesion area of $Adam8^{+/+}$ and $Adam8^{-/-}$ mice ($n = 9$ mice per group) after 10 weeks of WTD feeding (nonparametric Mann-Whitney U test). (e) mRNA expression levels in aortic arches of $Adam8^{+/+}$ and $Adam8^{-/-}$ ($n = 14/13$ mice) after 10 weeks of WTD (nonparametric Mann-Whitney U test).

Supplemental figure 4. Images of full-length blots of figure 2c

Chapter 6

Endothelial ADAM10 deficiency exacerbates murine atherosclerosis and is associated with intraplaque neovascularization and hemorrhage

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In preparation

Abstract

The transmembrane endopeptidase A Disintegrin And Metalloproteinase 10 (ADAM10) cleaves a range of membrane-bound proteins, including adhesion molecules, junctional molecules, cytokine, chemokine and growth factor receptors, a process referred to as ectodomain shedding. We previously showed that ADAM10 is upregulated in human atherosclerotic arteries, while its expression is associated with plaque progression and neovascularization. Here we investigated the role of ADAM10 in atherogenesis *in vivo*. Genetic deletion of endothelial *Adam10* (*Adam10^{ecto}*) in mice, rendered atherogenic by adeno-associated virus-mediated PCSK9 overexpression, markedly increased atherosclerotic lesion formation. Additionally, ADAM10 deficiency was associated with reduced plaque macrophage content, while granulocyte and collagen contents were unchanged. Strikingly, plaques of *Adam10^{ecto}* mice were hallmarked by intraplaque microvessels and hemorrhages, suggestive of major plaque destabilization. Collectively, these results demonstrate that endothelial ADAM10 is atheroprotective, most likely by limiting pathological neovascularization and inflammation.

Introduction

Atherosclerosis is a lipid-driven chronic inflammatory disease, which manifests in regions with disturbed flow in the medium- and large-sized arteries. The initiation of atherosclerosis is characterized by endothelial cell activation and dysfunction, leading to the disruption of endothelial barrier function and the active recruitment of leukocytes into the vessel wall ¹.

Limited surface proteolysis of transmembrane proteins, also referred to as (ectodomain) shedding, is increasingly recognized as a novel mechanism to regulate cell signaling and subsequent cellular responses. Shedding of numerous cell surface proteins is mediated by members of the A Disintegrin And Metalloproteinase (ADAM) family ². One of the prominent ADAM family members, ADAM10, was previously reported to be implicated in several physiological and pathological processes ^{2,3}. It is ubiquitously expressed in all vascular cells and has an important role in cardiovascular development ^{4,5}.

ADAM10 has a repertoire of different protein substrates, many of which involved in the pathogenesis of atherosclerosis, including lectin-like oxidized low-density lipoprotein receptor 1 (LOX-1) and receptor for advanced glycation end products (RAGE) ^{6,7}. Additionally, ADAM10 can regulate endothelial permeability and leukocyte transmigration by cleaving vascular endothelial cadherin (VE-Cadherin) ^{8,9}. Moreover, ADAM10 has been shown to limit sprouting angiogenesis and pathological neovascularization in developing mouse retina ¹⁰⁻¹². Previously, we have shown that ADAM10 is expressed in human atherosclerotic arteries, including in the endothelium, at levels that correlated with plaque progression and neovascularization ⁹. However, whether endothelial ADAM10 is causally involved in the pathogenesis of atherosclerosis remains to be determined.

Here, we report that endothelial ADAM10 deficiency aggravates atherosclerotic plaque formation in mice rendered atherosclerosis-prone by adeno-associated virus (AAV)-mediated proprotein convertase subtilisin/kexin type 9 (PCSK9) gene transfer. Moreover, while plaque granulocyte and collagen content remained unchanged, lesions of mice with ADAM10 deficiency had lower macrophage content and larger necrotic cores. Strikingly, lesions from ADAM10 deficient mice contained intraplaque microvessels and hemorrhage, features that are in general virtually absent in murine atherosclerotic lesions, already after 12 weeks of western type diet in relatively young mice ¹³.

Results

Endothelial ADAM10 deficiency increases atherosclerotic lesion size

To investigate the role of endothelial ADAM10 in atherosclerosis, endothelial ADAM10 wildtype (*Adam10^{wt}*) and knockout (*Adam10^{cko}*) mice were rendered atherogenic via AAV aided murine PCSK9 gene transfer, followed by WTD feeding for 12 weeks. As expected, both groups had increased plasma cholesterol and triglyceride levels upon WTD shift, however there was no difference between the genotypes (**fig. 1a and b**). Additionally, endothelial ADAM10 deficiency had no effect on leukocyte numbers in the blood before or after 12 weeks of WTD feeding (**suppl. fig. 1a**). Compared to *Adam10^{wt}* mice, aortic root plaque formation was significantly increased (+45%) in endothelial ADAM10 deficient mice (**fig. 1c**). Histological characterization of the atherosclerotic plaques showed that these lesions were more progressed in *Adam10^{cko}* mice compared to wildtype mice (**fig. 1d**). In addition, plaque expansion in *Adam10^{cko}* mice was associated with a decrease in relative macrophage content (**fig. 1e**), albeit at absolute level, lesional macrophage area was similar to wildtype mice (**suppl. fig. 1b**). In contrast, granulocyte (**fig. 1f**) and collagen (**fig. 1g**) contents were unchanged between both genotypes.

Atherosclerotic lesions of *Adam10^{cko}* mice display several plaque destabilizing features

Further examination of the atherosclerotic plaques in the aortic root revealed that *Adam10^{cko}* mice displayed several plaque destabilizing features, such as a significantly enlarged necrotic area (**fig. 2a**). Additionally, lesions of *Adam10^{cko}* mice were rich in IPH and neovessels, features that are rarely observed in murine aortic root lesions at this time point (12 weeks WTD, **fig. 2b-f**). IPH, defined as extravasated erythrocyte depositions in the intima (**fig. 2b, c and f**), was more frequently observed in *Adam10^{cko}* mice (62% of mice), while none of the wildtype mice carried intraplaque hemorrhages (**table 1**). The extent as well as location of IPH were variable, with hemorrhages located near the abluminal side of the intima (**fig. 2b-e**) as well as more closely to the luminal side (**fig. 2f**). Microvessels were 6x more frequent in aortic root plaques of *Adam10^{cko}* compared to wildtype mice (38% vs 6%, respectively; **table 1**). Of note, intimal microvessels appeared to be functional, as judged by the overt presence of intraluminal erythrocytes (**fig. 2d and e**). In addition, *Adam10^{cko}* mice also contained clusters of CD31⁺αSMA⁺ cells within atherosclerotic lesions of the aortic root, which were absent in wildtype mice (**fig. 2g and h; table 1**). Furthermore, there was no indication for any large vessel remodeling, based on left carotid artery morphology (**suppl. fig. 2c**).

Figure 1: Adam10^{ecko} mice have larger atherosclerotic lesions containing less macrophages. (a) Plasma cholesterol and (b) triglyceride levels of endothelial ADAM10 deficient (Adam10^{ecko}) and wildtype mice (Adam10^{wt}) mice rendered hyperlipidemic by AAV8-PCSK9 gene transfer, followed by 12 week western type diet feeding (n = 13-16). (c, d) Atherosclerotic lesion area (c) and plaque stage quantification (d) were analyzed in H&E-stained aortic root cross sections (H&E, n = 13-15; scale bar, 400 μ m). Representative examples of (immuno)histological stainings for macrophages (e, MAC3⁺; n = 12-14; scale bar, 200 μ m), granulocytes (f, Ly6G⁺; n = 13-16; scale bar, 100 μ m) and collagen (g, Sirius Red; n = 13-16; scale bar, 200 μ m) in aortic root atherosclerotic plaques with quantification. Mann-Whitney test was conducted to determine the statistical significance between the groups. ** P $\leq$ 0.01.

Figure 2. Aortic root plaques of *Adam10^{ecko}* mice on a western type diet have larger necrotic cores and show overt intraplaque neovascularization and hemorrhage.

(a) Quantification of necrotic core area ($n = 13-16$). (b) Atherosclerotic lesion in the aortic root containing pronounced intraplaque hemorrhage and microvessels of an *Adam10^{ecko}* mouse (H&E, scale bar, 400 μ m). l, lumen; m, media. (c) Immunofluorescence staining of erythrocytes (TER-119, red) in the atherosclerotic intima (cell nuclei, DAPI, blue) of an adjacent slide shown in (b; scale bar, 100 μ m). Lesion borders are indicated with dashed lines. (d) Detail of multiple small microvessels containing erythrocytes (indicated with dashed lines and black arrowheads) in aortic root lesion (H&E, scale bar, 200 μ m). (e) Detail of a large microvessel containing erythrocytes (indicated with dashed lines and black arrowhead) and intraplaque hemorrhage (indicated with white arrowhead) at the plaque base in the aortic root (H&E, scale bar, 50 μ m). (f) Atherosclerotic lesions containing intraplaque hemorrhage (indicated with white arrowheads) possibly originating from neovessels from the luminal side of the aorta (indicated with black arrowheads; H&E, scale bar, 100 μ m). (g, h) Clusters of cells (indicated with black arrowheads) within an atherosclerotic lesion of an *Adam10^{ecko}* mouse (H&E, scale bar, 100 μ m), which are positive for CD31 (blue, indicated by black arrowheads) and negative for α -smooth muscle actin (brown; scale bar, 100 μ m). Mann-Whitney test was conducted to determine the statistical significance between the two groups. *** $P \leq 0.001$.

Table 1: Characteristics of intraplaque hemorrhage, microvessels and CD31⁺ cell clusters in aortic root of *Adam10^{wt}* and *Adam10^{ecko}* mice after 12 weeks on western type diet. Fisher's exact test was conducted to determine the statistical significance between the two groups. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

Number of mice with intraplaque	<i>Adam10^{wt}</i>	<i>Adam10^{ecko}</i>	P-value
Hemorrhages	0/16 (0%)	8/13 (62%)	0.0003 ***
Microvessels	1/16 (6%)	5/13 (38%)	0.0097 **
CD31 ⁺ α SMA ⁻ cell clusters	0/16 (0%)	4/13 (31%)	0.0301 *

Discussion

Our data are the first to establish that endothelial ADAM10 deficiency significantly exacerbates atherosclerotic lesion formation and promotes plaque vulnerability at very high frequency in mice.

Observational studies have identified IPH as a critical factor in necrotic core expansion and concomitant atherosclerotic plaque growth¹⁴. A major source of IPH is the expansion of a network of immature blood vessels within the intima of a plaque, originating either from the arterial lumen or the adventitial vasa vasorum¹⁵. Intraplaque microvessels may provide a major portal for lipids, leukocytes and cholesterol-rich erythrocytes into the atherosclerotic plaque, thereby promoting plaque inflammation and progression¹⁶⁻¹⁸. Intraplaque microvessels and hemorrhage were seen to appear in aged (>40 weeks) atherosclerosis-prone mice¹⁷, combined with a prolonged WTD feeding (>40 weeks)¹⁹, surgical²⁰, pharmacological (focal mast cell activation²¹), gene therapeutic manipulation (p53 gene transfer²²) or haploinsufficiency of a key extracellular matrix component (fibrillin-1)²³ at distal sites of the aorta, including the brachiocephalic and carotid artery. Surprisingly, in this study, endothelial ADAM10 deficiency increased intraplaque neovascularization and hemorrhage in the aortic root of relatively young mice (22-24 weeks), already after 12 weeks of WTD feeding. Moreover, these plaque destabilizing features may have contributed to the necrotic core enlargement. In addition, the lower lesional macrophage content may well be the resultant of the rapid necrotic core expansion in endothelial ADAM10 deficient mice, especially since the absolute macrophage content was similar between both genotypes.

Angiogenesis, the sprouting of new blood vessels from the existing vasculature, is a tightly regulated process that becomes aberrantly activated during pathological conditions, including atherosclerosis²⁴. Endothelial cells become angiogenic and expand into atherosclerotic lesions induced by various stimuli, including pro-angiogenic growth factors, like vascular endothelial growth factor (VEGF), hypoxia, metabolic stress and inflammation¹⁵. Murine whole-body and endothelial-specific deletion of ADAM10 was already reported to result in vascular abnormalities during embryonic development, caused by a hyper-angiogenic response in liver, heart, kidney and intestines^{4,5,10}. We are the first to report massive neovascularization under chronic inflammatory conditions, in the atherosclerotic plaque upon genetic ablation of ADAM10 in endothelial cells. Additionally, several lesions from endothelial ADAM10 deficient mice contained CD31⁺αSMA⁺ cell clusters resembling hyperplastic endothelial cells previously observed in the intestines and kidneys of *Adam10^{ecto}* mice¹⁰. ADAM10 has been shown to regulate the angiogenic process through shedding of various key receptors, including VEGF receptor 2 (VEGFR2) and Notch^{9,12,25}. Notch activation in endothelial cells limits tip cell selection and sprout formation, thereby reducing excessive sprouting and branching²⁶. Interestingly, interference with Notch signaling phenocopies the vascular abnormalities caused by ADAM10 deletion under baseline conditions¹⁰⁻¹², suggesting that Notch may also be involved in the pathological neovascularization in atherosclerotic lesions caused by endothelial ADAM10 deficiency.

Next to angiogenesis, ADAM10 also plays a role in inflammatory processes through shedding of various transmembrane molecules involved in endothelial permeability and leukocyte recruitment. Cleavage of transmembrane CX₃CL₁ and CXCL16 transforms these adhesion molecules into soluble chemokines for attracting leukocytes²⁷. In addition, ADAM10-mediated processing of VE-cadherin and junctional adhesion molecule A

increases endothelial permeability and leukocyte transmigration^{8,9,28}. Altogether, genetic ablation of ADAM10 in endothelial cells would in theory result in increased leukocyte adhesion but reduced transmigration and thus reduced atherosclerosis development, which is not the case in this study. However, an increased amount of surface adhesion molecules might result in an impaired de-adhesion of endothelial bound leukocytes, eventually leading to enhanced endothelial damage and vascular permeability, despite having more junctional molecules present. Additionally, absence of ADAM10 may also result in elevated surface levels of the pro-inflammatory receptors LOX-1 and RAGE, both being activated by ligands that are abundantly present during hyperlipidemic conditions, thereby enhancing the overall inflammatory phenotype of endothelial cells^{6,7}. Additionally, aside from its role in angiogenesis, Notch signaling is also involved in limiting inflammatory gene programs in endothelial cells^{29,30}, and thereby protects against atherosclerosis²⁹. It remains, however, to be determined whether ADAM10 deficiency enhances atherogenesis via its role in neovascularization, inflammation or both.

Taken together, the current study establishes endothelial ADAM10 as a protective factor in atherosclerosis in mice, most likely by limiting pathological neovascularization and inflammation. To what extent disrupted endothelial ADAM10 expression is a contributory factor in vulnerable plaque formation and plaque rupture in humans, remains to be determined.

Materials and Methods

Animals. Mouse experiments were approved by the Animal Ethics Committee of Maastricht University, the Netherlands (permit number 2013-009), and were performed in compliance with the Dutch government guidelines. Female endothelial specific (*Tie2-Cre*) *Adam10* knockout (*Adam10^{cko}*) and wildtype (*Adam10^{wt}*) littermate control mice on a mixed genetic background were previously described¹⁰ and generously provided by Dr. A. Ludwig.

Atherosclerotic lesion induction and analysis. Female wildtype and *Adam10^{cko}* mice (aged 10 to 12 weeks; *n* = 16 and 14, respectively) were rendered prone to atherosclerosis by a single intravenous injection of adeno-associated virus serotype 8 containing D377Y-murine PCSK9 (AAV-PCSK9; 1×10^{11} vector genomes per mouse; as described previously³¹), followed by western type diet (WTD) feeding (0.25% cholesterol; Special Diets Services, Witham, Essex, UK). Blood was collected from the tail vein for analyses of plasma lipids at baseline (before WTD) and after 1, 3, 6, 9 and 12 weeks of WTD feeding after 4 hours fasting. After 12 weeks of WTD feeding, mice were anesthetized, euthanized and perfused with PBS containing nitroprusside (0.1 mg/ml, Sigma). Mouse hearts were excised and fixed overnight in 1% paraformaldehyde. Serial paraffin sections of the aortic root were cut (4 μ m) and stained with hematoxylin and eosin (H&E, Sigma) for morphometric analysis of lesion size, plaque, necrotic core area (defined as acellular regions) and plaque phenotype staging. Total plaque areas were obtained by averaging measurements of five representative H&E sections (20 μ m apart) of the aortic root. Plaque phenotype characterization was determined as previously described³², with slight modifications. Plaques were classified as early (foam cell rich, but lacking a necrotic core), moderately advanced (containing a fibrotic cap and often a necrotic core, but no medial macrophage infiltration) and advanced lesions, typified by medial macrophage infiltrates, elastic lamina degradation and more pronounced necrosis and fibrosis. Atherosclerotic lesions were further characterized for macrophage (MAC3, clone M3/84, 1:200, BD), granulocyte (Ly6G, clone 1A8, Becton & Dickinson; BD) and collagen (Sirius Red) content and microvessels (CD31, clone MEC13.3, 1:25, BD) and alpha smooth muscle actin (α SMA; clone 1A4, 1:3000, Sigma). Cell nuclei were counterstained with hematoxylin. Immunofluorescent staining of erythrocytes in atherosclerotic lesions was performed using anti-TER-119 antibodies (1:500, Biolegend) and cell nuclei were stained with DAPI. Pictures were taken using a Leica DM3000 light microscope and sections were analyzed in a blinded manner using computerized morphometry (Leica QWin V3, Cambridge, UK). Necrotic core was defined as cell and nucleus free plaque area, containing cholesterol clefts.

Flow cytometry analyses. Absolute circulating leukocyte subset numbers were determined by flow cytometry calibrated using Trucount Beads (BD, New Jersey, US). Blood was collected at the start (*t* = 0) and after 12 weeks of WTD. Erythrocytes were removed by incubation with erylisis buffer (155 mM NH_4Cl and 10 mM KHCO_3). Leukocytes were defined as CD45^+ (Biolegend), T-lymphocytes as $\text{CD45}^+ \text{CD3}^+$ (eBioscience) NK1.1^- (BD), NK cells as $\text{CD45}^+ \text{CD3}^- \text{NK1.1}^+$, B-lymphocytes as $\text{CD45}^+ \text{CD3}^- \text{NK1.1}^- \text{B220}^+$ (BD) granulocytes as $\text{CD45}^+ \text{CD3}^- \text{NK1.1}^- \text{B220}^- \text{CD11b}^+$ (BD) Ly6G^+ (BD) and monocytes as $\text{CD45}^+ \text{CD3}^- \text{NK1.1}^- \text{B220}^- \text{CD11b}^+ \text{Ly6G}^-$. Data were acquired using a FACS Canto II (BD Bioscience) and analyzed with FACSdiva software (BD Bioscience).

Blood lipid analyses. Blood was collected at the start (*t* = 0) and after 1, 3, 6, 9 and 12 weeks of WTD. Plasma was separated by centrifugation (2100 $\times$ g, 10 minutes, 4°C), and

stored at -80°C until further use. Plasma cholesterol and triglycerides were determined using standard enzymatic kits (Cholesterol FS'10; Triglycerides FS 5' Ecoline; Diagnostic Systems GmbH, Holzheim, Germany) according to the manufacturer's instructions.

Statistics. Data are presented as mean $\pm$ SEM. All statistical analyses were performed using the Prism software (GraphPad Software version 5, San Diego, CA, USA). Differences between two groups was evaluated for statistical significance with the Mann-Whitney test or Fisher's exact test. *P* values of less than 0.05 were considered significant.

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Supplemental figures

Supplemental figure 1: Endothelial ADAM10 deficiency does not affect blood leukocyte levels, absolute lesional macrophage content and large vessel morphology.

(a) Blood counts of several leukocyte subsets at baseline (t = 0; n = 10 per genotype) and after 12 weeks (t = 12; n = 10 per genotype) of western type diet (WTD). (b) Quantification of absolute lesional macrophage content in the aortic root (MAC3⁺ cells; n = 12-14) after 12 weeks of WTD. (c) Morphometric analysis of left carotid vessels of Adam10^{wt} and Adam10^{cko} mice.

Chapter 7

SPPL2-mediated intramembrane proteolysis controls pro-atherogenic signaling of LOX-1

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Abstract

Lectin-like oxidized low-density lipoprotein receptor 1 (LOX-1) is an established key player in the development of atherosclerosis by facilitating the uptake of oxidized low-density lipoprotein as well as inducing pro-atherogenic signaling. Besides being transcriptionally regulated, little is known about how LOX-1 function is controlled. Here, we show that LOX-1 function is regulated by multiple proteolytic post-translation modifications. Ectodomain shedding by the metalloprotease A Disintegrin And Metalloproteinase 10, resulting in the production of soluble LOX-1, and endo-lysosomal degradation generate membrane-bound N-terminal fragments (NTFs), which we identified as novel substrates of the intramembrane proteases signal peptide peptidase-like 2a and b (SPPL2a/b). Pharmacological inhibition or genetic deletion of SPPL2a/b results in the accumulation of LOX-1 NTFs, which in turn can amplify LOX-1-dependent pro-atherogenic signaling. Consequently, non-hematopoietic SPPL2a/b-deficient mice develop larger and more advanced atherosclerotic plaques than controls. Collectively, these results identify intramembrane proteolysis by SPPL2a/b as novel anti-atherogenic mechanism to suppress LOX-1-dependent signaling.

Introduction

Lectin-like oxidized low-density lipoprotein receptor 1 (LOX-1) is a multiligand scavenger receptor, which plays an important role in the development of atherosclerosis¹⁻⁴. LOX-1 is a type II transmembrane glycoprotein belonging to the family of C-type lectin receptors⁵. Originally identified as a major receptor of oxidized low-density lipoprotein (oxLDL) in endothelial cells, LOX-1 has also been found to be expressed by smooth muscle cells, fibroblasts, macrophages, dendritic cells, B-cells, platelets and adipocytes⁶. LOX-1 expression is relatively low under normal conditions⁷, however it can be markedly upregulated by a variety of pro-atherogenic stimuli, including pro-inflammatory cytokines, apoptotic bodies, advanced glycation end products and oxLDL *in vitro*⁶. Indeed, LOX-1 has been found to be upregulated in human atherosclerotic lesions⁸. Mouse models in which LOX-1 has been genetically deleted^{1,2} or overexpressed^{3,4} resulted in the attenuation or exacerbation of atherosclerosis development, respectively. In addition to facilitating oxLDL uptake, LOX-1 triggers various signaling pathways, including activation of mitogen-activated protein (MAP) kinases⁹ and the transcription factor NF- κ B¹⁰, resulting in the upregulation of pro-inflammatory cytokines and adhesion molecules, thereby promoting atherosclerosis development¹¹⁻¹⁴.

Like many cell surface proteins with a single-pass transmembrane domain, LOX-1 can undergo post-translational modification by proteolysis, leading to the release of its ectodomain into the extracellular space¹⁵. Circulating levels of the soluble form of this receptor (sLOX-1) have been found to be elevated in patients with acute coronary syndrome¹⁶. However, the proteolytic enzyme(s) responsible for the production of sLOX-1 have remained controversial^{15,17,18}. Furthermore, the biological function of the individual cleavage fragments and the impact of proteolytic processing on LOX-1-mediated signaling are undefined to date.

Proteolytic post-translational modification is a well-established mechanism to control the abundance and function of transmembrane proteins¹⁹. After the initial cleavage, in which the substrate's ectodomain is released (also referred to as ectodomain shedding), a second cleavage, which is facilitated by intramembrane-cleaving proteases (I-CLiPs), occurs within the transmembrane domain of the residual membrane-embedded stub, resulting in the release of the intracellular domain (ICD) into the cytosol.

Signal-peptide-peptidase-like 2a and b (SPPL2a, SPPL2b) are aspartyl I-CLiPs that can cleave N-terminal fragments (NTFs) derived from type II-oriented transmembrane proteins²⁰. Both SPPL2 proteases exhibit divergent subcellular localizations, i.e. whereas SPPL2a is targeted to late endosomes/lysosomes, SPPL2b is located at the plasma membrane²¹⁻²³. Although SPPL2a has been shown to be essential in B cell and dendritic cell development *in vivo*²⁴⁻²⁶, a physiological role for SPPL2b remains to be determined.

Here, we report a two-step proteolytic process regulating LOX-1 signaling function. In the first step, A Disintegrin And Metalloproteinase (ADAM) 10 and endo-lysosomal proteases act in concert to generate membrane-bound LOX-1 NTFs, with the capacity to potentiate LOX-1-mediated signal transduction. Second, as we demonstrated, the accumulation of these fragments are controlled by the I-CLiPs SPPL2a/b. Non-hematopoietic deficiency of SPPL2a/b exacerbates atherosclerosis development in mice, underpinning the importance of SPPL2a/b-mediated proteolysis in regulating LOX-1 pro-atherogenic functions.

Results

LOX-1 is processed by ADAM10 and by pH-dependent endo-lysosomal proteases

Based on previous reports of a soluble form of LOX-1 (sLOX-1), we aimed to investigate the posttranslational proteolytic processing of this protein in more detail. After we transiently expressed N-terminally hemagglutinin (HA)-tagged murine LOX-1 in HeLa cells (**fig. 1a**) or immortalized murine aortic endothelial cells (iMAEC; **fig. 1b**), we observed full-length LOX-1 protein as well as two hitherto unknown C-terminally truncated fragments, termed NTF1 and NTF2, at apparent molecular weights of approximately 25 and 17 kDa, respectively. The diffuse band of NTF1 suggests different degrees of glycosylation of the fragment. To confirm this, we substituted the two N-glycosylation sites N72 and N92 in the stalk domain (**suppl. fig. 1**) by alanine (**fig. 1c**). Mutation of N72 in HeLa cells led to a shift of NTF1, but not of NTF2. In contrast, blocking glycosylation at N92 did not alter the electrophoretic migration of any of the NTFs. This indicates that NTF1 and NTF2 represent distinct proteolytic fragments. Whereas NTF1 is generated by proteolysis between N72 and N92, the cleavage leading to NTF2 occurs between the N-terminus and N72.

Based on previous reports^{17,18}, we assessed the role of ADAMs in LOX-1 processing by inducing ectodomain shedding using ionomycin or 12-myristate-13-acetate (PMA), two established activators of ADAM10 and ADAM17, respectively²⁷. The release of sLOX-1 was strongly induced by ionomycin, but not PMA (**fig. 1d**). Furthermore, the production of sLOX-1 was completely abolished in human embryonic kidney (HEK) 293 cells lacking ADAM10, while the absence of ADAM17 had no influence on this fragment (**fig. 1e and f**). However, absence of ADAM10 only led to a minor reduction of both NTFs, despite its role in the processing of the extracellular portion of LOX-1, thus arguing for the involvement of additional proteolytic pathways.

To identify the class of proteases responsible for NTF production, we screened a panel of protease inhibitors. The cysteine protease inhibitor E64d caused a strong reduction of NTF2, while stabilizing NTF1 (**fig. 1g and h**). Mammalian cysteine proteases are primarily located in the cytosolic and endo-lysosomal compartments²⁸. To further distinguish between the two compartments, we blocked proteolytic activity within the endo-lysosomes using an inhibitor of endo-lysosomal acidification, bafilomycin A1. This reduced the levels of NTF1 and almost entirely depleted the smaller NTF2 (**fig. 1g and h**), which pointed towards a role, but not exclusively, for pH-dependent endo-lysosomal proteases in LOX-1 processing. In agreement, we observed a partial co-localization of transiently expressed LOX-1 with the lysosomal protein LAMP2 (**fig. 1i**).

Figure 1. LOX-1 N-terminal fragments are generated by ADAM10 and endo-lysosomal proteases.

Aside from the full-length LOX-1 protein (FL), two LOX-1 N-terminal fragments (NTF) were detected by western blot analysis upon overexpression of HA-mLOX-1 in either HeLa (**a**) or immortalized murine aortic endothelial cells (iMAEC; **b**) cells through detection of the HA-epitope fused to the N-terminus of the protein. (**c**) The potentially glycosylated residues N72 and N92 in the LOX-1 stalk region were individually mutated to alanine. Electrophoretic migration of full-length LOX-1 and NTFs was compared by western blot analysis (anti-HA) of transiently transfected HeLa cells. (**d**) iMAEC cells stably expressing HA-mLOX-1-FLAG were pre-incubated for 2 h in serum-free DMEM containing 10 μ M marimastat to block metalloprotease activity or DMSO as control. Then, cells were treated for 30 min with 1 μ M ionomycin or 100 nM PMA to activate ADAM10 or ADAM17, respectively. Whereas the full length receptor (FL) and the two NTFs were visualized by analysis of cell lysates using anti-HA, shed soluble LOX-1 (sLOX-1) was detected in trichloroacetic acid precipitates of conditioned media with an antibody against the LOX-1 ectodomain. (**e, f**) HEK293 cells deficient for ADAM10 (A10^{-/-}), ADAM17 (A17^{-/-}) or both metalloproteases (dKO) and wildtype cells (Wt) were transiently transfected with HA-mLOX-1-FLAG and subsequently cultivated in serum-free DMEM for 16 h. Cell lysates and conditioned media were analyzed as in (**d**). Mean values from a densitometric quantification $\pm$ SD of $n = 3$ independent experiments are shown in (**f**). (**g, h**) HeLa cells were transiently transfected with HA-mLOX-1 and subsequently treated overnight with either 100 nM bafilomycin A1 (baf A1), 1 mM pepstatin A, 40 μ M E64d, 1 μ M aprotinin, 10 μ M GM6001 or DMSO as solvent control. Differences in the formation of NTF1 (black arrow) and NTF2 (grey arrow) were finally visualized by western blot analysis using anti-HA antibodies. Mean values from a densitometric quantification $\pm$ SD of $n = 1 - 3$ independent experiments are shown in (**h**). (**i**) Delivery of LOX-1 to lysosomal compartments was visualized by indirect immunofluorescence in HeLa cells transiently transfected with HA-mLOX-1 and treated with either DMSO as control or 100 nM baf A1 for 6 h. While LOX-1 was detected using an HA-directed antibody, lysosomal compartments were stained with anti-LAMP2 (scale bar, 10 μ m).

LOX-1 N-terminal fragments undergo intramembrane cleavage by SPPL2a/b

Following ectodomain shedding, many residual membrane-bound protein stubs are further subjected to intramembrane proteolysis¹⁹. We therefore hypothesized that the LOX-1 NTFs could be further processed by I-CLiPs. Based on their topology, which predisposes them to cleave single type II oriented transmembrane proteins, and subcellular localization, we considered SPPL2a and SPPL2b as potential candidate proteases^{21,22,29}. Co-expression of LOX-1 with either proteases (**fig. 2a**), but not their catalytically inactive mutants (D/A), was associated with a reduction of both NTFs that could be blocked with the established SPP/SPPL inhibitor (Z-LL)₂-ketone³⁰. However, NTF depletion induced by SPPL2b was consistently more pronounced (**fig. 2b**). Upon SPPL2a or SPPL2b co-expression, a smaller band presumably representing the proteolytically released LOX-1 ICD was detected (**fig. 2a**). Furthermore, immunofluorescence analysis confirmed overlapping distributions between the substrate and both proteases (**fig. 2c**).

We further investigated whether LOX-1 NTFs are also substrates of endogenous SPPL2a and SPPL2b proteases. In LOX-1 expressing iMAEC cells, (Z-LL)₂-ketone significantly stabilized both LOX-1 NTFs (**fig. 2d and e**). Similarly, analysis of LOX-1 processing in wildtype and SPPL2a/b double-deficient (SPPL2a/b dKO) murine embryonic fibroblast (MEF) cells showed that steady-state levels of both NTFs were significantly higher in the knockout cells, indicating a reduced turnover (**fig. 2f and g**). To delineate the individual contributions of SPPL2a and SPPL2b, we also determined LOX-1 processing in SPPL2a (SPPL2a^{-/-}) and SPPL2b (SPPL2b^{-/-}) single-deficient MEF cells. Though a minor increase of the LOX-1 NTF1 was observed in SPPL2b^{-/-} MEFs, the combined ablation of SPPL2a and SPPL2b led to an even higher accumulation of both LOX-1 NTFs (**fig. 2h**), suggesting a synergistic effect of endogenous SPPL2a/b in LOX-1 processing.

To confirm that the effect of SPPL2a/b on LOX-1 NTFs represents proteolysis within their transmembrane segment, we expressed a LOX-1 model NTF (**fig. 2i**) comprising amino acids 1-88 together with SPPL2a or SPPL2b in HEK293 cells and recovered the C-terminal cleavage products for mass-spectrometric analysis from the media. To avoid mass heterogeneity by N-glycosylation, we mutated the confirmed N-glycosylation site at N72. We observed six specific cleavage fragments (peak no. 1-6, **fig. 2j**), which were not detected in samples from cells where either transfection of the substrate or the proteases had been omitted or the SPP/SPPL inhibitor (Z-LL)₂-ketone was concurrently applied (**suppl. fig. 2**). The determined peaks were matched with theoretical masses calculated for C-terminal peptides based on the NTF sequence (**fig. 2k**). Both proteases exhibited multiple, overlapping cleavage sites within the transmembrane segment of LOX-1. Interestingly, SPPL2a showed a strong preference for proteolysis N-terminal to L52 (peak no. 3), whereas the predominant cleavage site used by SPPL2b was shifted by one residue to I53 (peak no. 2).

Figure 2. LOX-1 NTFs undergo SPPL2a/b-dependent intramembrane proteolysis.

(a) HeLa cells were transiently transfected with HA-mLOX-1 and wildtype or catalytically inactive (D/A) murine SPPL2 proteases. Where indicated, SPPL2a/b protease activity was inhibited with 20 μ M (Z-LL)₂-ketone (ZLL) for 6 h prior to western blot analysis using anti-HA, anti-mSPPL2a or anti-mSPPL2b. Positions of the LOX-1 full-length protein (FL), NTF1, NTF2 and the released LOX-1 ICD are indicated. (b) Quantification of $n = 4$ independent experiments as shown in (a). * $P \leq 0.05$, ** $P \leq 0.01$ (One way ANOVA with Dunns post-test) (c) Co-localization of HA-mLOX-1 and either murine SPPL2a-myc or SPPL2b-myc was demonstrated in transiently transfected HeLa cells by indirect immunofluorescence detecting the HA or myc epitopes.

Figure 2 continued. LOX-1 NTFs undergo SPPL2a/b-dependent intramembrane proteolysis.

(**d, e**) Endogenous SPPL2a/b protease activity was inhibited in iMAEC cells transiently transfected with HA-mLOX-1 by 40 μ M ZLL for 24 h prior to Western blot analysis (anti-HA). LOX-1 NTF1+2 to full length ratios were calculated from 5 individually treated samples in $n = 2$ experiments are depicted in (**e**) as mean $\pm$ SD. * $P \leq 0.05$ (Student's t -test). (**f, g**) Wildtype (Wt) or SPPL2a/b double-deficient (dKO) MEFs were stably transduced with HA-mLOX-FLAG followed by western blot analysis (anti-HA, anti-mSPPL2a, anti-mSPPL2b). NTF/FL ratios are depicted in (**g**) as mean $\pm$ SD from $n = 3$ experiments. ** $p \leq 0.01$ (Student's t -test). (**h**) MEFs deficient for SPPL2a (2a^{-/-}), SPPL2b (2b^{-/-}) or both proteases (dKO) as well as wildtype controls were transiently transfected with HA-mLOX-1. Lysates were analyzed by western blot analysis (anti-HA, anti-mSPPL2a, anti-mSPPL2b). (**i-k**) Mass-spectrometric determination of the SPPL2a/b cleavage sites within the LOX-1 NTF. (**i**) Amino acid sequence of the employed model substrate consisting of aa 1-88 of LOX-1 with a N72A mutation (red) and an N-terminal V5 and a C-terminal FLAG tag. The determined cleavage sites of SPPL2a and SPPL2b within the transmembrane domain (grey) are marked by red arrows. (**j**) C-terminal fragments of V5-mLOX-1₁₋₈₈ N72A-FLAG secreted upon protease co-expression were purified from conditioned media and analyzed by mass spectrometry. Arrows indicate peptides upregulated by concomitant protease overexpression, with the predominant peaks labelled in red. (**k**) Table of peptides assigned to the respective peaks as shown in (**j**). obs., observed; calc., calculated. **potentially modified.

Endogenous LOX-1 is a substrate of SPPL2a and SPPL2b

To validate the proteolytic processing of endogenous LOX-1, we generated antiserum against an N-terminal epitope (aa 2-19) of the protein (**fig. 3a**). Additionally, we isolated primary murine aortic cells (pMAC) from aortae of wildtype and SPPL2a/b-deficient (*Sppl2a/b*^{dKO}) mice, which were validated by flow cytometry (**suppl. fig. 3**). We confirmed endogenous expression of SPPL2a and SPPL2b both in wildtype pMACs and aortic lysates (**fig. 3b**). Inhibition of SPP/SPPL proteases led to an accumulation of endogenous LOX-1 NTF1 and NTF2 in wildtype pMACs (**fig. 3c**). In agreement, both NTFs were present in pMACs from *Sppl2a/b*^{dKO} mice (**fig. 3d**). To exclude any bias due to the pMAC isolation, we analyzed LOX-1 processing directly in aortae isolated from wildtype as well as SPPL2a/b single- and double-deficient mice. Interestingly, in these samples we could only detect NTF2 (**fig. 3e**). Similar to the pMAC experiment, only small amounts of LOX-1 NTF2 were detected in wildtype tissue, whereas *Sppl2a/b*^{dKO} aortae exhibited a major accumulation of this fragment. In both the SPPL2a/b single-deficient aortae NTF abundance was mildly increased. However, levels were much lower than in the *Sppl2a/b*^{dKO} aortae, strongly highlighting the critical importance of both proteases for effective LOX-1 processing *in vivo*.

Figure 3. Endogenous LOX-1 is processed by SPPL2a and SPPL2b.

(a) To analyze the proteolysis of endogenous LOX-1, an antibody against the N-terminus of the protein was generated. Functionality of the affinity-purified antibody was evaluated by western blot analysis of either vector or HA-mLOX-1-transfected HeLa cells. As a control, the same lysates were probed with anti-HA antibody (3F10). Positions of the LOX-1 full-length protein (FL) and the two N-terminal fragments (NTF1, NTF2) are indicated. (b) Expression of SPPL2a and SPPL2b was demonstrated in wildtype (Wt) primary murine aortic cells (pMACs) and total aortic lysates by western blot analysis. To control for specificity of the employed antibodies, corresponding SPPL2a/b-deficient samples (dKO) were analyzed in parallel. (c) Wt pMACs were treated for 24 h with either 5, 10, 20 or 40 μ M (Z-LL)₂-ketone (ZLL) prior to western blot analysis of LOX-1 NTF accumulation using the newly generated LOX-1 antibody. (d) Wt or dKO pMACs were analyzed for LOX-1 NTF levels by western blot analysis applying the N-terminal antibody. (e) LOX-1 NTF levels were analyzed in aortae lysates from Wt, single deficient SPPL2a (2a^{-/-}) or SPPL2b (2b^{-/-}) as well as dKO mice by Western blotting.

Inhibition of SPPL2a/b enhances LOX-1-induced signaling responses

To address the impact of SPPL2a/b-mediated LOX-1 proteolysis on *bona fide* pro-atherogenic signaling of this receptor, we treated doxycycline inducible LOX-1 HEK293 cell line (**suppl. fig. 4a**) with the LOX-1 ligand oxLDL (**fig. 4a**). This triggered phosphorylation of the MAP kinases ERK1/2 (pERK) in LOX-1 expressing cells, while this was not observed in doxycycline untreated cells in which LOX-1 expression was absent. Importantly, inhibition of SPPL2a/b led to strongly enhanced LOX-1-dependent ERK activation. This effect was recapitulated in inhibitor-treated non-transfected iMAEC cells (**fig. 4b**) as well as in pMACs lacking these I-CLiPs (**fig. 4c**). These data further substantiate that intramembrane proteolysis by SPPL2a/b negatively regulates LOX-1 signaling.

Since SPPL2a/b inhibition or deficiency was accompanied by LOX-1 NTF accumulation (**fig. 3b-d**), we considered a role of these fragments in the regulation of LOX-1 functions. Therefore, we stably expressed an untagged LOX-1 NTF in iMAECs (**fig. 4d and e**) and pMACs (**suppl. fig. 4b**) and determined ERK phosphorylation in response to oxLDL in these as well as GFP-transduced control cells. In both types of NTF-expressing cells, ERK activation was strongly augmented, thereby phenocopying the effects seen upon inhibition (**fig. 4a and b**) or deficiency (**fig. 4c**) of SPPL2a/b. LOX-1 signaling activates multiple inflammatory pathways in vascular cells, including the upregulation of intracellular adhesion molecule 1 (ICAM-1)^{3,11}. This prompted us to investigate whether ICAM-1 levels would be influenced by SPPL2a/b proteases and/or the LOX-1 NTFs. We observed a significant upregulation of ICAM-1 both in SPPL2a/b-deficient and LOX-1 NTF overexpressing pMACs as compared to control cells (**fig. 4f and g**). These findings strongly suggest that SPPL2a/b deficiency via an accumulation of LOX-1 NTFs induces a pro-atherogenic state in vascular cells characterized by an enhanced response to oxLDL and upregulation of adhesion molecules. Interestingly, LOX-1 NTF coimmunoprecipitated with the full-length LOX-1 receptor, suggesting that a molecular interaction between the NTF and the full-length LOX-1 protein may augment LOX-1 signaling (**suppl. fig. 4c**).

Figure 4. Intramembrane proteolysis by SPPL2a/b negatively regulates LOX-1-dependent signaling. (a) LOX-1 expression was induced using doxycycline (dox) in stably transfected T-rex Flp-In cells. Prior to stimulation with 40 μ g/ml oxLDL, cells were cultured in serum-free DMEM for 4h in presence of DMSO or 40 μ M (Z-LL)₂-ketone (ZLL). Phosphorylated and total ERK1/2 levels were determined by western blot analysis. (b) iMAECs were incubated in presence of 40 μ M ZLL or DMSO for 24 h. For the last 4h, the cells were transferred to serum-free DMEM prior to treatment with 40 μ g/ml oxLDL and analysis of ERK1/2 activation. (c) Wildtype (Wt) or SPPL2a/b-deficient (dKO) pMACs were cultured for 4h in serum-deprived medium and treated with 40 μ g/ml oxLDL or left non-stimulated. ERK1/2 activation was determined by western blot analysis. (d) Generation of iMAEC cells stably expressing an untagged LOX-1 N-terminal fragment (NTF, aa 1-88) or eGFP as control. LOX-1 NTF and GFP expression were validated by western blot and fluorescence microscopy (not shown), respectively. (e) The cell lines characterized in (d) were cultivated for 4h under serum-free conditions prior to stimulation with 40 μ g/ml oxLDL and assessment of MAPK-activation. (f) Expression of ICAM1 was compared in Wt or dKO pMACs by western blot analysis. (g) Wt pMAC cells were transiently transfected with the LOX-1₁₋₈₈ NTF. Cells were analyzed for endogenous ICAM-1 expression by western blot. Band intensities of ICAM-1 were quantified densitometrically and normalized to actin levels in the respective samples, which were compared between LOX-1 NTF- and GFP-expressing cells. Bars indicate mean values $\pm$ SD of 6 individually transfected samples from two independent experiments. ** $P \leq 0.01$ (Student's *t*-test).

Non-hematopoietic SPPL2a/b deficiency augments atherosclerosis development, despite reduced plasma cholesterol levels

Based on our *in vitro* observations, we reasoned that SPPL2 and SPPL2b play a protective role in atherosclerosis development. Therefore, we first investigated whether SPPL2a and SPPL2b are expressed in human atherosclerotic plaques. Immunostaining of human carotid atherosclerotic plaques showed expression of both proteases in lesional endothelial cells and foam cells (**suppl. fig. 5a**).

To determine the impact of SPPL2a/b on atherosclerosis, we used bone marrow cells from wildtype mice to reconstitute the bone marrow of lethally irradiated wildtype ($Wt \rightarrow Wt$) and $Sppl2a/b^{dKO}$ ($Wt \rightarrow Sppl2a/b^{dKO}$) mice, to exclude the confounding effect of the immunocompromised phenotype caused by a defective processing of CD74 in $Sppl2a/b^{dKO}$ mice^{23,24}. Reconstitution with wildtype bone marrow rescued the previously described B-cell maturation defect in $Sppl2a/b^{dKO}$ mice (**suppl. fig. 5b and c**). After a recovery period, we rendered the bone marrow reconstituted mice prone to atherosclerosis by adeno-associated virus-mediated overexpression of a proprotein convertase subtilisin/kexin type 9 (PCSK9) gain-of-function mutant, followed by western type diet (WTD) feeding for 9 weeks.

Surprisingly, the $Wt \rightarrow Sppl2a/b^{dKO}$ mice did not gain any weight over a period of 9 weeks of WTD feeding (**fig. 5a**). Furthermore, both groups had increased plasma triglyceride and cholesterol levels upon WTD feeding, however, non-hematopoietic SPPL2a/b deficiency resulted in significantly reduced plasma cholesterol levels after 3 (-28%), 6 (-33%) and 9 (-38%) weeks of WTD compared to $Wt \rightarrow Wt$ mice (**fig. 5b and c**). In contrast, plasma triglyceride levels were similar between both genotypes.

Strikingly, despite having lower plasma cholesterol levels, atherosclerosis development was significantly increased by $\pm 41\%$ in the aortic root of $Wt \rightarrow Sppl2a/b^{dKO}$ mice compared to control mice after 9 weeks of WTD (**fig. 5d**). Histological characterization of the atherosclerotic lesions revealed an increased proportion (52% vs. 36%) of advanced atherosclerotic plaques in $Wt \rightarrow Sppl2a/b^{dKO}$ mice compared to controls (**fig. 5e**), which coincided with an increased necrotic core size (**fig. 5f**). Whereas there was no change in macrophage (**fig. 5g**) or smooth muscle cell content (**fig. 5h**), plaques of non-hematopoietic SPPL2a/b deficient mice were significantly enriched in collagen (**fig. 5i**). Furthermore, in aortae excised from $Wt \rightarrow Sppl2a/b^{dKO}$ mice after 9 weeks of WTD, ERK1/2 phosphorylation was significantly enhanced compared to $Wt \rightarrow Wt$ mice (**fig. 5j and k**), while ERK1/2 phosphorylation was unchanged in both Wt and $Sppl2a/b^{dKO}$ mice on chow diet (**suppl. fig. 5d**). Moreover, in agreement with our *in vitro* data, ICAM-1 expression was also significantly upregulated in the aortae from non-hematopoietic SPPL2a/b deficient mice (**fig. j and k**). These data strongly suggest SPPL2a/b deficiency associated enhancement of LOX-1 signaling as underlying mechanism.

Figure 6. Non-hematopoietic SPPL2a/b deficient mice have larger atherosclerotic lesions with a more advanced phenotype and display augmented pro-atherogenic signaling.

Atherosclerosis was induced in wildtype (Wt) and SPPL2a/b-deficient mice which received a Wt bone marrow, hereafter indicated as *Wt*→*Wt* and *Wt*→*Sppl2a/b*^{ΔKO}, respectively, by adeno-associated virus-mediated overexpression of murine PCSK9 combined with a western type diet (WTD). (a) Bodyweight, plasma triglycerides (b) and cholesterol (c) was measured at baseline (0) and after 3, 6 and 9 weeks of WTD (*n* = 18-20). ***P* ≤ 0.01, ****P* ≤ 0.001 (Student's *t*-test). (d-f) Atherosclerotic lesion area (d), plaque stage quantification (e) and necrotic core area (f) were analyzed in H&E-stained aortic root cross sections of *Wt*→*Wt* (*n* = 18) and *Wt*→*Sppl2a/b*^{ΔKO} (*n* = 15) after 9 weeks of WTD (scale bars, 400 μm). **P* ≤ 0.05 (Student's *t*-test). (g-i) (Immuno)histochemical staining of aortic root lesions for MAC3⁺ macrophages (g; *n* = 13-17), αSMA⁺ smooth muscle cells (h; *n* = 13-18) and Sirius Red stained collagen (i; *n* = 15-19). Scale bars, 200 μm. ****P* ≤ 0.001 (Student's *t*-test), values are represented as mean ± SEM. (j, k) Phosphorylation of ERK1/2 and expression of ICAM-1 were quantified in aortic arch lysates from *Wt*→*Wt* and *Wt*→*Sppl2a/b*^{ΔKO} after 9 weeks of WTD by western blot analysis (*n* = 5-6 per genotype). Values are normalized to *Wt*→*Wt* and are represented as mean ± SD. ***P* ≤ 0.01 (Student's *t*-test).

Discussion

In this study, we show that LOX-1 is subject to multiple proteolytic post-translational modifications. Notably, LOX-1 processing by ADAM10 and pH-dependent endo-lysosomal serine proteases generates NTFs which enhance pro-atherogenic LOX-1 signaling. Further processing of NTFs by the I-CLiPs SPPL2a and SPPL2b accelerates clearance, resulting in reduced NTF aided LOX-1 signaling. *In vivo*, non-hematopoietic deletion of both SPPL2 proteases in mice led to aggravated atherosclerosis development, despite lower plasma cholesterol levels.

Previous studies have suggested phenylmethylsulfonyl fluoride-sensitive serine proteases¹⁵ or the metalloproteinases ADAM17¹⁸ or ADAM10¹⁷ to mediate ectodomain shedding of LOX-1. Our data support a predominant role for ADAM10, but not ADAM17, in the proteolytic liberation of sLOX-1, though, we cannot rule out the possibility that additional sheddases may be employed by cells depending on specific biological stimuli. Moreover, in addition to ectodomain shedding, we report here a so far unrecognized endo-lysosomal pathway of LOX-1 processing with far-reaching implications on receptor stability and its downstream functions. Based on NTF production, this endolysosomal pathway exerts a relatively higher contribution to the overall LOX-1 turnover than ADAM10-mediated ectodomain shedding under steady-state conditions. This might be due to the distribution of LOX-1 between the cell membrane and endo-lysosomal compartments, the accessibility of the protease to its substrate as well as the abundance and activity of the proteases, all of which are most likely in favor for the endo-lysosomal proteases.

Despite its fundamental role in atherosclerosis¹ and extensive studies linking LOX-1 with a variety of cellular responses^{9-14,31}, the proximal molecular events leading to these downstream responses are still poorly defined. Several interaction partners have been identified, including the adaptor protein Rho-associated protein kinase 2 (ROCK2)³², however it remains enigmatic how LOX-1 with its short cytoplasmic domain of 31 amino acids devoid of any canonical signaling motifs triggers signaling cascades upon binding of its ligands⁵. Importantly, LOX-1 NTFs as intermediate degradation products can influence LOX-1-mediated signal transduction, however the mechanism still remains an outstanding question. Like many C-type lectins, oligomerization of LOX-1 is required for high affinity binding of its ligand oxLDL³³. The LOX-1 NTFs, devoid of a ligand binding domain, were found to associate with the full-length LOX-1 receptor, suggesting that a region between the cleaved stalk region and N-terminus may contain an oligomerization motif. The association between full-length LOX-1 and its NTFs may provide additional sites for the recruitment of adaptor proteins, like ROCK2, thereby lowering the signaling threshold of the receptor upon ligand binding. In addition, LOX-1 NTFs might increase the surface expression of full-length LOX-1 by facilitating its trafficking to the plasma membrane or by impeding ectodomain shedding by preventing close proximity between ADAM10 and the full-length LOX-1 receptor. Lastly, the LOX-1 NTF might also induce a conformational change in full-length LOX-1 leading to optimal ligand binding and/or receptor signaling.

To prevent their accumulation, clearance of the short membrane-embedded LOX-1 NTFs is dependent on intramembrane proteolysis by SPPL2a/b, which generates a soluble ICD. However, whether the LOX-1 ICD is functionally inert or has any signaling capabilities remains to be determined. Except with LOX-1 overexpression, we were unable to detect any endogenous LOX-1 ICDs under steady-state conditions, which may be reflected by low rates of LOX-1 proteolysis, rapid turnover of the released ICD and/or a limited sensitivity of our LOX-1 antibody and does not necessarily preclude a biological function.

In the present work, we demonstrated that SPPL2a/b deficiency in the non-hematopoietic compartment aggravated atherosclerosis development in mice. However, the role of SPPL2a/b in atherosclerosis may even be underestimated. First of all, due to the bone marrow transplantation, several LOX-1 expressing subsets of leukocytes^{5,34}, including macrophages, which are a major constituents of atherosclerotic plaques and were shown to express both SPPL2a and SPPL2b (**suppl. fig. 5a**), were not deficient for both SPPL2 proteins. Although the functionality of these cells may be modulated by other SPPL2a/b substrates like CD74, blocking LOX-1 proteolysis also in macrophages may enhance LOX-1 signaling and thus further promote the progression of atherosclerotic lesions. Secondly, in spite of their unique phenotype upon WTD feeding, characterized by the absence of any increase in bodyweight and a ± 28 -38% reduction in plasma cholesterol levels, it is quite remarkable that non-hematopoietic SPPL2a/b deficient mice developed larger and more advanced atherosclerotic lesions. In addition, these results unveil a role for SPPL2a/b in the regulation of cholesterol metabolism and body weight maintenance under hypercholesterolemic conditions.

The enhanced MAPK signaling and ICAM levels in the atherosclerotic lesions of mice with stromal SPPL2a/b deficiency are in agreement with our *in vitro* findings. Furthermore, plaques had a higher collagen content, reflecting the pro-fibrotic activity of LOX-1, which is not limited to atherosclerosis², but has also been observed upon cardiac remodeling following ischemia^{35,36} and different models of renal injury³⁷⁻³⁹. Therefore our findings strongly suggest impaired LOX-1 proteolysis as the predominant underlying mechanism in atherosclerosis development of non-hematopoietic SPPL2a/b-deficient mice. However, we cannot exclude that SPPL2a and SPPL2b fulfil additional biological functions that could likewise lead to the observed phenotype.

In summary, we have identified post-translational proteolysis as a major regulatory mechanism for LOX-1 pro-atherogenic signaling. Degradation of LOX-1 NTFs by SPPL2a/b prevents their intracellular accumulation and suppresses NTF-mediated augmentation of LOX-1 signaling. Furthermore, we have established the SPPL2a/b-LOX-1 axis in stromal cells as a atheroprotective factor.

Materials and Methods

Experimental animals. Generation of SPPL2a/b double-deficient mice has been previously described by Schneppenheim *et al.*²³. Breeding of mice, animal care and handling were performed in accordance with local and national guidelines (V 242.7224.121-3). Mice were housed in individually ventilated cages in the animal facility of the Christian-Albrechts-University Kiel, Germany or Maastricht University, the Netherlands. The atherosclerosis experiment was approved by the Animal Ethics Committee of Maastricht University, the Netherlands (permit number 2014-097).

Plasmids. Expression constructs encoding wildtype murine SPPL2a and SPPL2b fused to a C-terminal Myc epitope or their catalytically inactive mutants D416A or D414A as well as corresponding constructs for human SPPL2a were described before^{22-24,40}. C-terminal Myc-tagged constructs encoding either wildtype or catalytically inactive (D421A) human SPPL2b were derived from previously described cDNAs⁴¹ and inserted into pcDNA3.1/Hygro+ vectors (Thermo Scientific) using HindIII and XhoI restriction sites. The open reading frame coding for murine LOX-1 was amplified from a LOX-1 expression plasmid (a kind gift of Tatsuya Sawamura, National Cerebral and Cardiovascular Center, Japan). The PCR product comprising a N-terminally appended HA epitope was ligated into pcDNA3.1/Hygro+ via BamHI and XbaI restriction sites. Based on this plasmid, glycosylation mutants of murine LOX-1 receptor were generated from this plasmid by mutating N72 and N92 to alanine.

For nuclear translocation assays, α -HA-mLOX-1 and α -NLS-HA-mLOX-1 plasmids with the N-terminally fused β -galactosidase alpha peptide were generated according to Mentrup *et al.*⁴², where also the employed α -eGFPnuc, α -TMEM192 β GEFC and Firefly luciferase assay and/or transfection control plasmids were described. A GFP-encoding pMSCV puro plasmid was a kind gift of Michael Engelke, University of Göttingen. Untagged murine full length LOX-1 or the putative murine LOX-1 NTF consisting of amino acids 1-88 of the receptor were cloned into the pMSCV puro vector (Clontech) using BglII and XhoI restriction sites. Correspondingly, a HA-mLOX-1-FLAG construct in this vector was generated. The coding sequence of human LOX-1 was obtained from Sinobiological, fused to an N-terminal HA tag and cloned into pcDNA3.1/Hygro(+) via BamHI and XbaI restriction sites. For mass spectrometric determination of the SPPL2a/b cleavage sites in human and murine LOX-1 NTFs, coding sequences of NTFs comprising amino acids 1-88, with appended N-terminal V5- and C-terminal FLAG epitopes were inserted into pcDNA3.1/Hygro+. In these constructs, the glycosylated asparagine residues N72 (murine) and N73 (human) were mutated to alanine to reduce complexity of mass spectrometric analysis.

Cell culture and transfection. HeLa cells (DSMZ), Flp-In™ T-REx™ 293 (Invitrogen), HEK293 ADAM10/17 knockout cells, Murine embryonic fibroblasts (MEFs) and Platinum-E retroviral packaging cells (Cell Biolabs, Inc.) were cultivated in DMEM (GIBCO) supplemented with 10 % fetal calf serum (FCS; Biochrom) as well as 100 U/ml penicillin and 100 μ g/ml streptomycin (both Sigma). ADAM10 and ADAM17 single- and double-deficient HEK293 cells were generated by CRISPR/Cas9 genome-editing and as described by Riethmueller *et al.*⁴³. Immortalized murine aortic endothelial cells

(iMAEC) were a kind gift of Prof. Hanjoong Jo, Atlanta, USA and cultured as described by Bond *et al.* ⁴⁴.

MEFs were isolated from 13.5 day old embryos from wild type, *SPPL2a*^{-/-}, *SPPL2b*^{-/-} and *SPPL2a/b* double-deficient mice. After removing head and internal organs, embryos were cut into small pieces and incubated in trypsin-EDTA for 15 min at 37°C. The resulting tissue pieces were dissociated by pipetting and the cell suspension transferred to 12 ml pre-warmed DMEM supplemented with 10% FCS and antibiotics. Cells were centrifuged, washed once in fresh medium and plated out for culture. For immortalization, MEFs were transfected at an early passage with an expression plasmid of the SV40 large T antigen (pMSSVLT) ⁴⁵. Immortalized cells were used for experiments after an average of 10 passages.

Primary murine aortic cells (pMAC) were isolated from thoracic aortic vessels of either wild type or *SPPL2a/b* double-deficient mice based on an explant strategy with blunt dissection of aortae ⁴⁶. In brief, CO₂-euthanised mice were injected with 4 ml of sterile PBS containing 10 U/ml heparin. Subsequently, the thoracic aorta was isolated and cleaned from perivascular fat and adventitial tissue. After opening the vessel longitudinally to expose the endothelial cell layer, the aorta was cut into small squares that were placed into gelatin-coated 24-well-plates to allow cell outgrowth. Aortic explants and the derived pMACs were cultivated in DMEM supplemented with 20 % FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml endothelial cell growth supplement from bovine neural tissue (Sigma) as well as 1x concentrated non-essential amino acids (GIBCO). All primary cells were used at low passage numbers. All cells were cultivated at 37 °C in a humidified 5% CO₂/air atmosphere.

For transient transfection of HeLa, iMAEC, MEF and Platinum-E cells, Turbofect transfection reagent (Thermo Scientific) was applied. Flp-In™ T-REx™ 293 cells stably expressing murine LOX-1 or the LOX-1 ICD constructs were generated by transfection with the indicated constructs and the pOG44 vector encoding for the Flp recombinase followed by selection with 100 µg/ml hygromycin and 10 µg/ml blasticidin (both Invivogen). After selection, surviving clones were combined and further analyzed as polyclonal cell lines. To induce transgene expression, 10 µg/ml doxycycline (Sigma) was added to the medium for 24h.

In LOX-1 expressing cell lines, LOX-1-dependent signal transduction was stimulated with 40 µg/ml oxidized low-density lipoproteins (oxLDL) obtained from Hycultec. To influence proteolytic processing of LOX-1, several compounds were added 24h prior to analysis to the medium of cultured cells, unless indicated otherwise. The lysosomal acidification inhibitor bafilomycin A1 (Sigma) was used in a final concentration of 100 nM. To inhibit cysteine, serine and metallo- proteases, E64d (40 µM, Enzo), Leupeptin (2.5 µM, Roth) and GM-6001 (10 µM, Calbiochem) were applied for 24h. The cell-permeable Pepstatin A-methyl ester (10 µM, Merck) was used to interfere with the activity of aspartyl proteases. Activity of the metalloproteinases ADAM10 and ADAM17 was blocked by marimastat (10 µM, Sigma) or stimulated by treatment with ionomycin (1 µM, Sigma) or PMA (100 nM, Sigma), respectively. SPPL2 proteases were inhibited by application of the SPP family inhibitor (Z-LL)₂-ketone (Peptanova) in concentrations ranging from 10 to 40 µM. Except for Leupeptin and E64-D, which were applied as aqueous solutions, all protease inhibitors were dissolved in dimethyl sulfoxide (DMSO)

which was used as solvent control in respective experiments.

Retroviral transduction. For generation of ecotropic retroviruses for transduction of iMAEC, MEF and pMAC cells, Platinum-E cells were transfected using Turbofect transfection reagent. After 48h, virus-containing supernatants were removed from Platinum-E cultures and passed through a 20 μ m filter (BD Biosciences). Subsequently, supernatants supplemented with 8 μ g/ml polybrene (Sigma) were added to iMAEC or pMAC cells seeded a day before in 10 cm culture dishes. To allow transduction of HEK293-based T-RexTM cells, Ecotropic Receptor booster (Clontech) was applied according to the manufacturer's instructions. One day after transduction, supernatants were replaced by fresh culture medium containing 10 μ g/ml puromycin (Invivogen). Stably transduced cells were maintained as polyclonal cell lines without sub-cloning.

Protein extraction and western blot analysis. Cell and tissue lysis was performed as described by Schneppenheim *et al.* ²⁴. In brief, cells were harvested by centrifugation and subsequently extracted in lysis buffer (50 mM Tris/HCl pH 7.4, 150 mM NaCl, 1.0% Triton X-100, 0.1% SDS) supplemented with complete protease inhibitor mix (Roche), Pepstatin A (0.5 μ g/ml, Sigma), 4 mM EDTA and Pefabloc[®] SC Protease Inhibitor (4 mM, Roth). Aortic vessels were flushed with PBS and cleaned from perivascular fat and adventitial tissue. For lysis, 150 μ l of lysis buffer and ceramic beads were added to aortae which were homogenized by vigorous shaking of the mixture in a Precellys homogenizer (Peglab). Afterwards, cell and tissue suspensions were sonicated using a Branson Sonifier 450 (Emerson Industrial Automation) and incubated at 4°C for 1h. The remaining debris was pelleted by centrifugation at 13,000 g for 10 min at 4°C and protein concentrations of supernatants were determined using the Pierce[™] BCA Protein Assay Kit (Thermo Scientific). After lysis, protein lysates were complemented with the required amount of reducing SDS-PAGE sample buffer according to Laemmli ⁴⁷ and denatured for 10 min at 56°C.

For recovery of soluble LOX-1 from conditioned media of HA-mLOX-1-FLAG expressing iMAEC or HEK293 cells, these were cultured in serum free medium for 30 min prior to stimulation with ionomycin or PMA for 30 min. After stimulation, 800 μ l supernatant were supplemented with 200 μ l 100% (w/v) trichloroacetic acid for 1 h at 4°C. Precipitated proteins were sedimented by centrifugation at 13000 g for 10 min at 4°C. The resulting pellet was washed once with ice cold acetone prior to resuspension in reducing SDS-page sample buffer and denaturation at 95°C for 10 min.

Electrophoretic separation by SDS-PAGE with a standard Tris-glycine buffer system ⁴⁷, semidry transfer to nitrocellulose membranes and immunodetection was conducted as previously described ⁴⁸. Monoclonal antibodies against HA (3F10), Myc (9B11), V5 and FLAG (M2) epitope tags were obtained from Roche, Cell Signaling or Sigma, respectively. Soluble LOX-1 was detected applying a polyclonal goat antibody directed against the C-terminus of this protein (AF1564, R&D systems). To allow detection of LOX-1 N-terminal fragments, rabbits were immunized with a synthetic peptide corresponding to the amino acids 2-19 of the protein (Pineda Antikörper Service). Similarly, a polyclonal antiserum against residues 575-592 of human SPPL2b was raised. In both cases, antisera were affinity-purified against the immobilized immunogens prior to use. Polyclonal

antibodies for detection of murine SPPL2a²², murine SPPL2b²³ and human SPPL2a⁴⁰ were described before. For detection of MAPK kinase activation, monoclonal antibodies against phosphorylated ERK1/2 (T202/Y204, D13.14.4E) as well as total ERK1/2 (137F5) from Cell Signaling were used. Murine ICAM-1 was detected with the monoclonal antibody YN1/1.7.4 from Biolegend. Primary antibodies were diluted in 5% milk powder dissolved in Tris-buffered saline supplemented with 0.1% (v/v) Tween-20 (TBST). For detection of phosphorylated ERK1/2 and p38, 5% BSA (w/v) (Roth) in TBST was used as antibody diluent. Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Dianova. Detection of chemoluminescent signals was performed with a LAS4000 imaging system (GE Healthcare), and intensities of the relevant protein bands quantified with ImageJ.

Immunoprecipitation. Cell lysates were prepared in IP buffer (50 mM Tris-HCl, 150 mM NaCl, 1% (w/v) Triton X-100, 4 mM EDTA supplemented with the same proteinase inhibitors as the standard cell lysis buffer described above. Incubation with primary antibodies was performed at 4°C overnight under continuous end-over-end rotation. The following day, 50 µl equilibrated Protein G Agarose beads (Thermo Scientific) were added and the incubation continued for 1h. Beads were recovered by centrifugation, washed five times with IP buffer and finally eluted by boiling in reducing SDS PAGE sample buffer.

Indirect immunofluorescence. Immunocytochemical stainings were performed as previously described⁴⁸. Briefly, cells grown on coverslips were fixed with 4% (w/v) paraformaldehyde. Lysosomal compartments were visualized with the 2D5 monoclonal LAMP-2 antibody⁴⁹. Alexa 488 and -594 conjugated secondary antibodies were purchased from Molecular Probes. Nuclei were stained with the DNA-binding DAPI (4',6-diamidino-2-phenylindole from Sigma-Aldrich) which was included in the embedding medium at a concentration of 1 µg/ml. Images were acquired with a Olympus FV1000 confocal laser scanning microscope (Olympus, Hamburg, Germany) and further processed with Olympus Fluoview software and Adobe Photoshop software.

Flow cytometry. FACS analysis of the isolated wildtype and SPPL2a/b double-deficient pMAC lines was facilitated by antibodies against murine ICAM-1 (YN1/1.7.4, Biolegend, APC) and VCAM-1 (429, Biolegend, PE) conjugated with PE or APC fluorophores, respectively. To assess the reconstitution of B cell development after bone marrow transplantation in the atherosclerosis experiment, B cells were measured in the blood and spleen. Cell suspensions were prepared from harvested spleens by passing the tissue through a 70 µm cell strainer (Greiner). Blood cells and splenocytes were incubated in erythrocyte lysis buffer (150 mM NH₄Cl, 10 mM NaHCO₃, pH 7.4) for 2 minutes on ice, washed and filtered through a 70 µm cell strainer. Subsequently, splenic cells were blocked with anti-CD16/32 (clone 93, eBioscience), washed and stained with antibodies against B220 (RA3-6B2, eBioscience, PE-Cy7), CD21 (8D9, eBioscience, PE) and CD24 (30-F1, eBioscience, APC). Blood cells were blocked with anti-CD16/32 (clone 93, eBioscience), washed and stained with antibodies against CD45 (Biolegend, 30-F11, PerCP) and B220 (RA3-6B2, eBioscience, PE-Cy7). Absolute cell numbers in blood were calculated

using Trucount tubes (BD Biosciences). Cells were analysed using a FACS Canto II (BD Biosciences) and FACSDiva software v6 (BD Biosciences) for data interpretation.

Nuclear translocation assay. To analyse nuclear translocation of the LOX-1 ICD, a reporter assay based on β -galactosidase (β -gal) enzyme fragment complementation (β -GEFC) was performed according to Mentrup *et al.* ⁴². Therefore, the HEK293T-based ω_{nuc} reporter cells, which stably express the nucleus-targeted β -gal ω -fragment, were seeded in 96 well-plates one day prior to transfection with plasmids encoding LOX-1 fusion proteins (α -LOX-1, α -NLS-LOX-1) carrying the β -gal α -peptide at the N-terminus. In parallel, the previously described positive (α -eGFP_{nuc}) and negative (α -TMEM192) assay controls were included ⁴². Additionally, cells were co-transfected with a Firefly luciferase-coding vector to control for differences in transfection efficiency and to allow normalization of the assay results. One day post transfection, cellular β -galactosidase and Firefly Luciferase (Luc) activities were determined with the Dual-Light® Luciferase & β Galactosidase Reporter Gene Assay System (Life technologies) and a GloMax™ 96 well plate luminometer E6521 (Promega). Based on these values, β Gal/Luc ratios were calculated.

Cleavage site determination. Epitope-tagged LOX-1 NTFs (V5-mLOX-1₁₋₈₈ N72A-FLAG, V5-hLOX-1₁₋₈₈ N73A-FLAG) were transiently expressed in T-REX™ 293 cells with or without stable overexpression of SPPL2a or SPPL2b. The C-peptides released by the SPPL2-mediated intramembrane cleavage were recovered from conditioned media employing anti-FLAG M2-conjugated agarose beads (Sigma). After repeated washing with IP/MS buffer (0.1% *n*-octyl glucoside, 10 mM Tris/HCl, pH 8.0, 5 mM EDTA and 140 mM NaCl) and sterile water, peptides were eluted by trifluoroacetic acid/acetonitrile/water (1:20:20, vol/vol/vol), saturated with α -cyano-4-hydroxycinnamic acid. The dissolved samples were dried on a stainless plate and subjected to MALDI-TOF-MS analysis using a VoyagerDE STR instrument (Applied Biosystems).

Bone marrow transplantation and atherosclerosis model. Two weeks prior to bone marrow transplantation, female *SPPL2a*^{-/-}*SPPL2b*^{-/-} and wild type mice were received antibiotics supplemented water (neomycin (100 mg/L; Gibco, Carlsbad, Ca USA) and Polymyxin B sulfate (60.000 U/L; Gibco)), which was continued for 5 weeks after the procedure. Mice were exposed to 6 Gy total body irradiation one day before and on the day of transplantation with 5×10^6 bone marrow cells isolated from male wildtype mice. After a recovery period of 5 weeks, hypercholesterolemia was induced by a single intravenous injection of adeno-associated virus serotype 8 containing D377Y-murine PCSK9 (AAV-PCSK9; 1×10^{11} vector genomes per mouse; as previously described ⁵⁰, followed by high-cholesterol diet (WTD) feeding containing 0.25% cholesterol (Special Diets Services, Witham, Essex, UK) for 9 weeks. Plasma concentrations of triglycerides and cholesterol were determined at baseline just before WTD initiation as well as after 9 weeks using standard enzymatic kits (Cholesterol FS'10; Triglycerides FS 5' Ecoline; Diagnostic Systems GmbH, Holzheim, Germany) according to the manufacturer's instructions. Prior to blood sampling from the tail vein, mice were fasted for 4 hours.

Histological analysis. After 9 weeks of WTD feeding, mice were anesthetized, euthanized and perfused with PBS containing nitroprusside (0.1 mg/ml, Sigma). Hearts were excised and fixed in 1% paraformaldehyde overnight. Serial paraffin sections of the aortic root were cut (4 μ m) and stained with hematoxylin and eosin (H&E, Sigma) for morphometric analysis of plaque size and necrotic core area (defined as acellular regions). Total plaque areas were obtained by averaging measurements of five representative (H&E) sections (20 μ m apart) of the aortic root. Plaque stages were determined as previously described⁵¹, with slight modifications. Plaques were classified as early (foam cell rich, but lacking a necrotic core), moderately advanced (containing a fibrotic cap and often a necrotic core, but no medial macrophage infiltration) and advanced lesions, typified by medial macrophage infiltrates, elastic lamina degradation and more pronounced necrosis and fibrosis. Atherosclerotic lesions were further analyzed for abundance of macrophages (MAC3, clone M3/84, 1:200, BD), smooth muscle cells (aSMA, clone 1A4, 1:3000, Sigma) and collagen content (Sirius Red, Polysciences). Sections (MAC3, aSMA) were subjected to heat-induced antigen retrieval using citrate buffer (pH6, DAKO), and specific antigen-antibody binding was visualized using appropriate biotinylated secondary antibodies, ABC horse-radish peroxidase (Vector Laboratories) and DAB substrate kit (DAKO). Cell nuclei were counterstained with hematoxylin. Pictures were taken using a Leica DM3000 light microscope (Leica Microsystems, Wetzlar, Germany) and sections were analyzed in a blinded manner using Adobe Photoshop CS6 software (Adobe, San Jose, US).

Immunohistochemistry of human plaque sections. The use of human carotid artery tissue for these studies was in agreement with the code of proper secondary use of human tissue in the Netherlands. Human carotid endarterectomy segments were fixed in paraformaldehyde and paraffin embedded. Sections were incubated with a polyclonal antisera against SPPL2a or SPPL2b (see section protein extraction), followed by detection with Brightvision secondary antibodies (ImmunoLogic) and Vector Red (Vector Laboratories). Cell nuclei were counterstained with hematoxylin. Pictures were taken on a Leica DM3000 light microscope (Leica Microsystems, Wetzlar, Germany) and sections were analyzed in a blinded manner using Adobe Photoshop CS6 software (Adobe, San Jose, US).

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Supplemental figure 3. Characterization of isolated primary mouse aortic cells.

Wildtype or SPPL2a/b-deficient (dKO) primary mouse aortic cells were analyzed for expression of endothelial marker proteins ICAM-1 and VCAM-1 by flow cytometry. The bars depicted show mean values of three independent measurements $\pm$ SD.

Supplemental figure 4. Validation of a doxycycline inducible LOX-1 overexpressing and LOX-1 NTF-overexpressing cell lines.

(a) T-Rex Flp In HEK293 cells with conditional overexpression of untagged LOX-1 full-length protein were generated and transgene expression was validated after induction with 10 μ g/ml doxycycline for 24h by western blot analysis using anti-LOX-1. (b) pMAC cells stably overexpressing the putative LOX-1 NTF comprising amino acids 1-88 of the LOX-1 protein or GFP as control were generated by retroviral transduction. Overexpression of LOX-1₁₋₈₈ (NTF) was demonstrated with an antibody directed against an N-terminal epitope of the receptor. To analyses LOX-1 signal transduction, cells were serum-deprived for 4 h prior to stimulation with 40 μ g/ml oxLDL for the indicated time points. MAPK activation was determined in total lysates by Western blotting using antibodies detecting either phosphorylated or total ERK1/2. (c) HeLa cells were transiently transfected with HA-tagged full-length LOX-1 (HA-mLOX-1) and a V5- and FLAG-tagged LOX-1 NTF (V5-mLOX-11-88-FLAG) alone or in combination. Interaction of both proteins was analyzed by co-immunoprecipitation using anti-V5. The NTF was detected with anti-V5 in cell lysates (Input) and the immunoprecipitates (IP). The full-length LOX-1 protein (FL) and its processing intermediates NTF1 and NTF2 were visualized with a horseradish peroxidase-conjugated HA-antibody.

Supplemental figure 5. SPPL2a and SPPL2b are expressed in human atherosclerotic lesions and reconstitution with wildtype bone marrow rescues SPPL2a/b-deficient mice from compromised B-cell maturation.

(a) Immunohistochemical staining of SPPL2a and SPPL2b (red) in human atherosclerotic lesions from patients who underwent carotid endarterectomy. Black and white arrowheads indicate endothelial cells (ECs) and foam cells, respectively. (scale bars overview lesion, 400 μ m; scale bars high power views lesions, 50 μ m). (b) After 9 weeks of western type diet, splenocytes from wildtype (Wt) or SPPL2a/b-deficient (*Sppl2a/b*^{dkO}) mice, which were reconstituted with Wt bone marrow, indicated as Wt → Wt ($n = 18$) and Wt → *Sppl2a/b*^{dkO} ($n = 18$), respectively, were assessed for B-cell maturation markers (B220, CD21 and CD24). In the representative dot plots viable B220⁺ cells are depicted and the proportion (%) of transitional stage 1 (T1) and 2 (T2) as well as mature B cells among viable splenocytes is shown. In addition, quantification of the transitional stages is shown. (c) blood B-cells were quantified using Trucount beads and flow cytometry before (0w) and after 9 weeks (9w) of WTD feeding ($n = 10$ per genotype per timepoint). Values are shown as mean $\pm$ SEM. (d) Levels of phosphorylated (pERK) or total ERK1/2 were determined by western blot analysis in aortae of either Wt or *Sppl2a/b*^{dkO} mice on chow diet ($n = 6$ per genotype). Bands were quantified by densitometry. Bars indicate mean values of pERK/ERK ratios normalized to that of wildtype $\pm$ SD.

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Chapter 8

General discussion

Main findings of this thesis

Atherosclerosis, the underlying cause of the majority of cardiovascular disease (CVD)-related deaths, is a vascular disease driven by an aberrant lipid metabolism and a maladaptive inflammatory response ¹. Although lipid lowering by statins has reduced the incidence of CVD events to some extent, there still remains a residual CVD risk that remains unresolved, even after aggressive/intensive statin therapy ². Therefore, there is a need for new therapeutics that restore the lipid imbalance and/or resolve the chronic inflammation in the atherosclerotic plaque.

Next to reducing plasma levels of low-density lipoprotein, the inverse relationship between high-density lipoprotein (HDL) cholesterol and CVD risk, together with the anti-atherogenic effects of HDL, has stimulated a great interest in therapeutically raising plasma HDL levels ³. In addition, post-translational proteolytic processing of transmembrane proteins, through ectodomain shedding by A Disintegrin And Metalloproteinases (ADAMs) or intramembrane proteolysis by signal peptide peptidase-like 2 (SPPL2) proteases, has emerged as a novel level of control of many biological processes, including immune responses ^{4,5}.

The overall aim of this thesis was to explore the (immuno)modulatory capabilities of HDL and the membrane-associated proteases ADAMs and SPPL2 in macrophage-mediated immune responses and atherosclerosis development, respectively.

The main findings of this thesis are that:

1. Contrary to vascular smooth muscle and endothelial cells, in macrophages HDL exert pro-inflammatory effects through passive cholesterol depletion and enhancement of toll-like receptor-mediated inflammatory pathways (**chapter 2**).
2. Ectodomain sheddases of the ADAM family have divergent effects on atherosclerosis development. While ADAM8 does not affect murine advanced atherosclerotic lesion progression, despite its upregulation in human atherosclerotic lesions (**chapter 5**), endothelial specific deletion of ADAM10 increases atherosclerosis development and is associated with intraplaque hemorrhage and neovascularization (**chapter 6**).
3. The intramembrane proteases SPPL2a/b limit LOX-1-mediated pro-atherogenic signaling and their deletion in the non-hematopoietic compartment, despite its cholesterol-lowering effect, enhances atherosclerosis development (**chapter 7**).

HDL (cholesterol) raising therapies - past failures, future opportunities?

Despite major advances in treatment of hypercholesterolemia, residual CVD risk still remains high in a significant number of patients. Early observational studies have consistently shown that HDL cholesterol (HDL-C) levels are inversely associated with both cardiovascular disease and mortality⁶⁻⁸. However, in recent years, several lines of evidence have emerged which suggest that simply raising HDL-C is insufficient in reducing cardiovascular risk. For instance, randomized clinical trials involving HDL-C-raising agents were prematurely terminated based on the lack of beneficial effects⁹ or even increased risk of mortality¹⁰. Furthermore, loss-of-function mutations in *LIPG* and *SCARB1* were associated with no change or even an increased CVD risk, respectively, despite being associated with high concentrations of plasma HDL-C^{11,12}. Based on these disappointing findings, it is postulated that plasma HDL-C concentration is not related to the cardioprotective effects of HDL. As a consequence, the HDL-C-centric view has shifted towards alternative indices of HDL, such as functionality, particle number and structure, subpopulation heterogeneity and distribution, and molecular composition¹³.

Cell-specific immunomodulation by HDL

The atheroprotective effects of HDL have been mainly attributed to its well-known role in the reverse cholesterol transport (RCT), in which excess cholesterol from the vessel wall is transported to the liver for excretion in bile¹⁴. However, HDL also has the capability to modulate inflammatory processes in cell types relevant to atherosclerosis development, including endothelial cells, vascular smooth muscle cells (VSMCs), monocytes and (lipid-laden) macrophages^{15,16}. Surprisingly however, HDL may modulate inflammation in a cell-type specific manner by dose-dependently exerting anti-inflammatory effects in endothelial cells, VSMCs, monocytes and macrophage foam cells, while augmenting pro-inflammatory processes in non-lipid-laden macrophages (**chapter 2**). In the setting of atherosclerosis, the anti-inflammatory effects of HDL seem to have the upper hand, since raising plasma HDL levels, either by HDL infusions or apolipoprotein A-I (apoA-I) overexpression, inhibits atherosclerotic plaque development in animals¹⁷⁻²¹ and regresses established lesions in both in animals²²⁻²⁴ and humans^{25,26}. From an inflammatory viewpoint, the atheroprotective effects of HDL can be mainly attributed to its ability to limit leukocyte recruitment by interfering with leukocyte-endothelial cell interactions and dampening of the overall inflammatory reaction in endothelial cells, VSMCs and lipid-laden macrophages¹⁵. However, whether the pro-inflammatory effects of HDL are beneficial to atherosclerotic plaque regression and stabilization remains to be determined. In general, pro-inflammatory processes have negative impact on atherosclerosis, since increased inflammation is associated with plaque destabilization²⁷. On the other hand, pro-inflammatory signaling, induced by apoA-I, was shown to be coupled to increased cholesterol efflux from macrophages²⁸, which is considered a key anti-atherogenic process²⁹. Furthermore, macrophage-mediated phagocytosis of apoptotic cells, termed efferocytosis, shows a high degree of overlap with microbial phagocytosis^{30,31}, which was found to be enhanced by HDL (**chapter 2**). However, in contrast to microbial phagocytosis, efferocytosis is generally not immunogenic and

elicits the production of anti-inflammatory/pro-resolving mediators, such as interleukin 10 and transforming growth factor β ³². Therefore, HDL might promote efferocytosis and consequently anti-inflammatory processes which may result in the suppression of atherosclerosis development. Therefore, although it might seem counterintuitive at first, the pro-inflammatory effects of HDL might aid actually in the protection against atherosclerosis.

In contrast, the anti-inflammatory properties of HDL, which are beneficial in protecting against atherosclerosis, may be detrimental in other cases where an adequate inflammatory reaction is warranted, such as bacterial infections. Therefore, as a counterbalance, HDL may prime the toll-like receptor response in macrophages for an effective bacterial clearance (**chapter 2**). However, whether this holds true for other types of infections, including viral and parasitic, and whether the pro-inflammatory effects of HDL are restricted to macrophages remains to be determined.

The complexity of high-density lipoproteins

HDL comprises a complex mixture of subpopulations that show considerable variation in size (7.2-12.9 nm diameter), shape (discoidal or spherical) and density (1.063-1.21 g/mL), which require specialized methods for determination (summarized in table 1)³³. Furthermore, HDL particles display a high degree of heterogeneity in terms of composition. Proteomic and lipidomic analyses have identified >80 different proteins and >200 individual lipid species in HDL^{34,35}. Differences in protein and lipid composition, and abundance affect the functionality of the HDL particle³⁵⁻³⁸. For example, many of the anti-atherogenic effects of HDL could be fully or partially attributed to sphingosine-1-phosphate, a biologically active lysosphingolipid associated to HDL, at least *in vitro*³⁹. Furthermore, the origin and type of phospholipids used to generate reconstituted HDL have been shown to have opposing effects in macrophages, in which soybean-derived phospholipids exert anti-inflammatory effects⁴⁰, while 1-palmitoyl-2-linoleoyl phospholipids exert pro-inflammatory effects when bound to apoA-I (**chapter 2**).

Additionally, HDL can carry multiple species of microRNAs thereby affecting gene regulation across different cell types and tissues⁴¹. To complicate matters even further, different metabolic and disease states can change the overall HDL particle composition or modify specific components, which in turn affect the functionality of the particle^{37,42-46}. For example, a rare naturally occurring mutant variant of the apoA-I protein, called apoA-I_{Milano}, has a higher antioxidant activity compared to wildtype apoA-I⁴⁷. On the other hand, disease-induced alterations of HDL, e.g. incorporation of serum amyloid A in inflammatory conditions, oxidation of apoA-I in atherosclerosis, glycation in type 2 diabetes and incorporation of symmetric dimethylarginine in chronic kidney disease, can render HDL dysfunctional or can even convert it into a pro-inflammatory particle

44,48-50.

Table 1: Classification of HDL subpopulations by physical properties (adapted from Rosenson *et al.*³¹⁾)

Methods	Distinctive physiochemical property	Very large HDL (HDL-VL)	Large HDL (HDL-L)	Medium HDL (HDL-M)	Small HDL (HDL-S)	Very small HDL (HDL-VS)
Density gradient ultracentrifugation (Density range, g/ml)	Density	HDL2b 1.063–1.087	HDL2a 1.088–1.110	HDL3a 1.110–1.129	HDL3b 1.129–1.154	HDL3c 1.154–1.170
Gradient gel electrophoresis (Size range, nm)	Surface charge	HDL2b 12.9–9.7	HDL2a 9.7–8.8	HDL3a 8.8–8.2	HDL3b 8.2–7.8	HDL3c 7.8–7.2
2-D gel electrophoresis (Size range, nm)	Surface charge and size	$\alpha 1$ 11.2–10.8	$\alpha 2$ 9.4–9.0	$\alpha 3$ 8.5–7.5	$\alpha 4$ 7.5–7.0	Pre- $\beta 1$ 6.0–5.0
Nuclear magnetic resonance spectroscopy (Size range, nm)	Spectral signals emitted from protons of terminal methyl groups of lipids present in HDL particles when subjected to a magnetic field	Large HDL-P 12.9–9.7	medium HDL-P 9.7–8.2	small HDL-P 8.2–7.2		
Ion mobility (Size range, nm)	Time of flight through a buffer gas	HDL2b 14.5–10.5	HDL2a and HDL3 10.5–7.65			

*HDL-P, HDL particle

While transporting cholesterol and other lipids between different tissues and lipoproteins, HDL particles undergo constant, dynamic remodeling⁵¹. Initially, binding of soluble apoA-I to ATP-binding cassette subfamily A1 (ABCA1) facilitates the efflux of cellular unesterified cholesterol and phospholipids, which associate with apoA-I to form small, lipid-poor, discoidal complexes termed pre- β 1 HDL. Additional uptake of cholesterol via ATP-binding cassette subfamily G1 (ABCG1) converts these complexes into small, discoidal α 4 HDL. After association with lecithin-cholesterol acyltransferase (LCAT), α 4 HDL is converted into larger spheroidal α 2 and α 3 HDL particles, by esterifying cholesterol and sequestering these highly hydrophobic cholesteryl esters into the particle's lipid core. Further remodeling through the activity of cholesteryl ester transfer protein (CETP), in which cholesteryl esters are exchanged for triglycerides (TG) from TG-rich lipoproteins, results in the formation of α 1 HDL. Moreover, hydrolysis and subsequent removal of phospholipids by hepatic lipase (HL), endothelial lipase (EL), and secretory phospholipase A2 (sPLA2) converts α 1 particles into α 2 HDL. Eventually, binding of HDL to scavenger receptor B1 (SRB1) facilitates the transfer of cholesteryl esters into the liver and steroidogenic tissues, after which the small, lipid-poor HDL detaches and repeats the above described cycle.

HDL subpopulations display differences in their composition and physiochemical properties, and thus may vary in their anti-atherogenic potential (extensively reviewed in Camont *et al.*⁵¹). For example, lipid-free/lipid-poor apoA-I and small, lipid poor pre- β 1 particles are the preferred acceptors of ABCA1-mediated cholesterol efflux, while larger HDL particles extract cholesterol more potently via ABCG1 and SRB1⁵². In contrast, cholesterol efflux via the receptor-independent passive diffusion pathway appears to be similar between the different HDL subpopulations. Interestingly, high levels of cholesterol depletion (>50% of total cellular cholesterol) results in substantial cell death, while limited cholesterol depletion (<50% of total cellular cholesterol) causes T cell activation⁵³, and secretion of pro-inflammatory mediators by adipocytes⁵⁴, *in vitro*. Therefore, the degree of cellular cholesterol depletion might give another explanation for the surprising dual effects of cholesterol-depleting agents (e.g. HDL) in macrophage inflammatory responses, as shown by de Nardo *et al.*⁴⁰ and our study (**chapter 2**). Furthermore, small, dense HDL particles have greater capacity to protect low-density lipoprotein (LDL) from oxidation and are more resistant to oxidative modification, while large, light HDL particles exert more potent vasodilatory and antithrombotic activities⁵⁵⁻⁵⁸. However, the potential heterogeneity in immunomodulatory activities by HDL subpopulations remain poorly characterized.

Current challenges for HDL-based therapeutics

Despite compelling evidence from preclinical animal studies demonstrating that HDL is atheroprotective, clinical trials of HDL-C-raising therapies failed to demonstrate a reduction in CVD events. This suggests that simply elevating HDL-C does not necessarily translate into improved HDL function. In fact, extreme high levels of HDL-C is associated with high mortality⁵⁹, warranting caution for elevating HDL beyond a certain threshold. In addition, perturbations in HDL biogenesis (apoA-I, ABCA1, ABCG1, LCAT), remodeling (CETP, HL, EL, sPLA2) or HDL content disposal

(SRB1) may adversely affect clinical outcome, despite the use of HDL-C-raising agents, as exemplified by a loss-of-function variant of the SRB1 gene which is associated with increased plasma HDL-C and, paradoxically, also with an increased risk of coronary heart disease ¹¹. Furthermore, little is known about which HDL functions are clinically relevant and which individual HDL subpopulations confer cardioprotection. Progress in these areas is being hampered, because it is proven problematic to isolate sufficient quantities of every HDL subpopulation and assess their functions, especially in humans. Therefore, future challenges will encompass the characterization of HDL subpopulations in terms of composition and functionality, especially in individuals with perturbed HDL metabolism. Furthermore, the development of novel HDL therapeutic interventions that specifically elevate plasma levels of beneficial HDL subpopulations with a favorable proteomic, lipidomic and genomic makeup and/or improve HDL functions relevant to atherosclerosis in a cell-specific manner will lower the number of cardiovascular events. In addition, the development of clinical assays to quantify clinically relevant HDL functions will aid in the risk stratification of cardiovascular disease in patients and in monitoring the efficacy of existing and novel treatment regimes.

Therapeutic targeting of ADAMs: in search of the right cell, protease and substrate in the right context

It is estimated that, under steady state conditions, up to 7% of all cell surface proteins are subjected to limited proteolysis, resulting in the release of their ectodomain in the extracellular space ⁶⁰. Altered expression and/or dysregulation of ADAMs can have profound effects on multiple cellular pathways, which may lead to the development or aggravation of a wide range of pathologies, including atherosclerosis ⁶¹. Multiple ADAMs are found to be upregulated in several diseases (**chapter 4**), including ADAM8 (**chapter 5**) and ADAM10 in atherosclerosis ⁶², however, due to their complex biology, it has proven difficult to develop therapeutic strategies that selectively targets an individual ADAM without any side-effects ⁶³.

Regulation of ADAMs

The activity of ADAMs is subject to multiple layers of regulation, including transcriptional control, alternative splicing, post-translational modifications, subcellular trafficking and localization, protein-protein interactions, the presence of natural inhibitors, and the abundance of both protease and substrate ⁶⁴.

Under steady state conditions, the abundance of catalytically active ADAMs on the cell surface is limited, however upon stimulation their surface expression can be rapidly increased ^{65,66}. For instance, the vast majority of both ADAM10 and ADAM17 is retained in the endoplasmic reticulum (ER), however by interacting with tetraspanins or iRhom1/2, respectively, triggers their maturation and trafficking towards the Golgi apparatus and cell surface ^{67,68}. ADAM-mediated shedding take place at the cell surface, however there is increasing evidence that ectodomain shedding can also occur in other cellular compartments, including in the Golgi apparatus and secreted exosomes ^{69,70}. Interestingly, in contrast to other proteases, the prevailing perception is that ADAM-mediated proteolysis is dependent on the specific conformation of the substrate and the distance of the juxtamembrane stalk, since ADAMs lack a well-defined consensus sequence for cleaving substrates ⁷¹⁻⁷³.

ADAMs are synthesized as zymogens containing an inhibitory pro-domain, which maintains these proteases in a latent state and for some ADAMs acts as a chaperone for proper folding ^{74,75}. The pro-domain is removed either in the trans-Golgi network or at the plasma membrane by proprotein convertases, like furin, thereby rendering the ADAMs catalytically active. Exceptions to the rule are ADAM8 and ADAM28, which activate themselves in an autocatalytic fashion ^{76,77}. Aside from their pro-domain, the shedding activity of most ADAMs can also be regulated by a family of endogenous inhibitors, called tissue inhibitors of metalloproteinases (TIMPs). Although ADAM8 is unaffected by TIMPs, TIMP1 can block ADAM10, while TIMP3 can inhibit both ADAM10 and ADAM17 activity ⁷⁸⁻⁸⁰. Additionally, integrins can negatively influence the proteolytic activity of ADAMs ⁸¹.

Furthermore, the shedding activity of both ADAM10 and ADAM17 has been shown to be regulated by the lipid composition of the plasma membrane. Cholesterol-enriched microdomains, or lipid rafts, work as specialized organizational units that regulate both the accessibility ADAMs to their substrates and their effective cleavage rates. Depletion

of membrane cholesterol enhances the activities of both ADAM10 and ADAM17⁸²⁻⁸⁴. Furthermore, statin treatment augments ADAM10 activity⁸³, while incubating endothelial cells with HDL enhances the activity of ADAM17 *in vitro*⁸⁵. However, whether several or all atheroprotective effects of HDL are mediated (partially) by ADAMs remains to be determined.

Comparable to lipid rafts, tetraspanins cluster together with other membrane proteins and lipids giving rise to the formation of higher-order lipid-protein membrane complexes termed tetraspanin-enriched microdomains⁸⁶. Tetraspanins of the TspanC8 subgroup have been shown to differentially regulate the subcellular localization and substrate preference of ADAM10 depending on the interacting TspanC8⁸⁷. Furthermore, the tetraspanin CD9 was shown to negatively regulate the proteolytic activity of ADAM17⁸⁸.

Functional redundancy among ADAM family members?

Humans and mice express 22 and 37 different ADAMs, respectively, 12 of which display metalloproteinase activity in both species⁶⁴. ADAMs have been shown to have an overlapping repertoire of substrates, at least *in vitro*, suggesting that there exists a certain amount of redundancy between the different ADAMs⁵. However, gene knockout studies in mice revealed that ADAMs have both redundant and non-redundant roles depending on the ADAM, substrate and the physiological context. Mice deficient in ADAM8, 9 and 15 do not display any overt phenotype, while whole body deficiency of either ADAM10 or ADAM17 results in embryonic lethality⁵. Despite their close relationship in terms of structure and substrate repertoire, ADAM10 and ADAM17 seem to have distinct roles *in vivo*. ADAM10 deficient embryos die at E9.5 with multiple defects in the developing central nervous system, somites and cardiovascular system, resembling those of Notch deficient mice⁸⁹. On the other hand, ADAM17 knockout mice die between E17.5 and the first day after birth with abnormalities in the developing heart, lung and epithelial tissues, similar to genetic deletion of the epidermal growth factor receptor or its ligands⁹⁰. Moreover, pharmacological inhibition or endothelial-specific deletion of ADAM17 results in reduced sprouting angiogenesis, pathological neovascularization and collateral formation, opposite to the cardiovascular effects of ADAM10⁹¹⁻⁹⁴. Additionally, ADAM17 hypomorphic mice on a low-density lipoprotein deficient background have enlarged atherosclerotic lesion formation⁹⁵. Surprisingly, however, the effects of ADAM17 on atherosclerosis development seem to be cell type-specific, since endothelial-specific knockout of ADAM17 reduces atherosclerotic plaque size, while myeloid ADAM17 deficiency increases lesion development⁹⁶. Opposite to ADAM17, myeloid ADAM10 deficiency shifts the atherosclerotic lesions towards a stable phenotype, characterized by increased collagen content⁹⁷, while genetic deletion of ADAM10 in endothelial cells aggravates atherosclerosis development (**chapter 6**). Collectively, these findings show that ADAMs can function in a cell type-specific manner, as well as refute functional redundancy of some ADAMs during development, adulthood and diseased states, like atherosclerosis. Additionally, ADAMs may also function in a context-dependent manner. For instance, next to its roles in atherosclerosis, ADAM10 has been shown to be protective in Alzheimer's disease, by preventing the production of the neurotoxic β -amyloid peptide, while having adverse effects in cancer by stimulating excessive proliferation and metastasis⁹⁸. Furthermore, despite being upregulated in both cancer

and atherosclerosis, blocking ADAM8 activity, either pharmacologically or genetically, respectively, only affects cancer progression ⁹⁹, while having no effect on atherosclerosis development (**chapter 5**).

ADAMs as therapeutic targets: still a long way to go

Based on accumulating evidence over the years, it has become clear that ADAMs play an important role in several diseases, including neurodegeneration, cancer and atherosclerosis, and are therefore attractive new targets for novel therapies ¹⁰⁰. However, several important aspects have to be taken under consideration before an effective ADAM-based intervention can be designed and implemented.

Besides their role in pathological conditions, ADAMs also support multiple physiological functions in healthy tissues and, as mentioned earlier, intervening with this tightly regulated network of proteases may have adverse effects. Indeed, loss of *ADAM17* causes severe multi-organ dysfunction in humans ^{101,102}, while upregulation of *ADAM10* may promote cancer development and progression ⁹⁸. In contrast, *ADAM8* does not seem to play a role during adult homeostasis, and is upregulated predominantly during various inflammatory diseases, making *ADAM8* an excellent target for intervention ¹⁰³.

Furthermore, given their pleiotropic effects, some ADAMs can be both harmful and protective, depending on the disease, the cell type and their substrates, as exemplified by *ADAM10*. Uncovering disease- and cell-specific sheddome of ADAMs and verifying the newly identified substrates *in vivo* will provide a better understanding of the physiological and pathological role of ADAMs. Special attention has to be given to disease-dependent post-translational modifications of ADAMs and their substrates, which, depending on the modification, may facilitate or even protect a transmembrane protein from cleavage ^{104,105}.

Moreover, clinical trials targeting the active zinc site in the metalloproteinase domain of the best studied ADAM protease, *ADAM17*, were discontinued due to lack of selectivity ⁶³, since all members of the metzincin family of proteases, which include among others matrix metalloproteinases, ADAMs and ADAMs with thrombospondin motifs, contain a Zn^{2+} ion in their catalytic domain ¹⁰⁶. Preventing this cross-inhibition of metalloproteinases will require the development of selective inhibitors that target other moieties than the active zinc site in the metalloproteinase domain. Targeting components involved in the trafficking and maturation of ADAMs (e.g. iRhoms or tetraspanins) or unique secondary sites (exosites), which reside outside of the substrate-binding pocket of ADAMs and participate in substrate recognition and binding, will provide new opportunities to modulate the outcome of a disease ¹⁰⁴.

SPPL2-mediated intramembrane proteolysis: a novel approach against cardiovascular diseases?

Unlike ADAM proteases, research on SPPL2 proteases is still in its infancy. Aside from SPPL2b being upregulated in the hippocampus of Alzheimer's disease patients ¹⁰⁷ and a single nucleotide polymorphism in the *SPPL2C* gene being associated with increased susceptibility to idiopathic pulmonary fibrosis in humans ¹⁰⁸, very little is known regarding the roles SPPL2 proteins play in human disease. Moreover, despite recent advances, we have only begun to scratch the surface of SPPL2 biology.

Regulation of SPPL2 proteins

Similar to ectodomain shedding, intramembrane proteolysis needs to be tightly regulated. SPPL2 proteins are under tissue specific transcriptional control. While SPPL2a protein is ubiquitously expressed, SPPL2b expression is limited to the brain, bone marrow and lymphoid tissues in decreasing order of expression in mice under steady-state conditions ¹⁰⁹. In addition, we have shown for the first time that both SPPL2a and SPPL2b are expressed in human atherosclerotic lesions. (**chapter 7**). Furthermore, a microarray analysis of human tissues showed that SPPL2c was expressed in the brain, heart, skin, adrenal cortex, mammary gland and testis ¹¹⁰. However, unlike other members of the SPPL2 subfamily, SPPL2c is still uncharacterized. Given the lack of introns and high degree of polymorphisms in the human gene, it has been suggested that SPPL2c is a recently evolved pseudogene ¹¹¹.

Next to transcriptional regulation, SPPL2 proteins display differential subcellular localization, which influences their access to substrates. After being synthesized at the ER, SPPL2a is transported to late endosomes and lysosomes, while SPPL2b is located at the cell membrane, in which the majority resides in lipid rafts ¹¹²⁻¹¹⁴. However, upon overexpression, large quantities of SPPL2a were also detected at the cell membrane ^{112,113}, while SPPL2b was also present in the Golgi apparatus and endolysosomal vesicles ^{115,116}. Though sorting of SPPL2a is facilitated by a C-terminal tyrosine-based sorting motif ¹¹², the signal(s) involved in SPPL2b trafficking are unknown. Furthermore, it remains to be determined whether the subcellular localizations of SPPL2a and SPPL2b change during different pathophysiological conditions.

In addition to the localization of both protease and substrate in the same cellular compartment, proteins have to fulfill certain requirements to be able to be subjected to intramembrane proteolysis by SPPL2 proteases. While both SPPL2a and SPPL2b solely accept type II oriented transmembrane proteins ^{110,117}, to date, however, substrate requirements have mainly been investigated for SPPL2b. Ectodomain removal of a protein, either by sheddases like ADAMs or endolysosomal proteases, is a prerequisite for the subsequent intramembrane proteolysis by SPPL2b and consequently is the rate-limiting step ¹¹⁷. In addition, analogous to ADAMs, intramembrane proteolysis is based on the secondary structure of the substrate, since SPPL2b does not cleave at a specific consensus sequence within the substrate's transmembrane or juxtamembrane domains ¹¹⁸. However certain sequence determinants within these domains are required for efficient intramembrane proteolysis by SPPL2b ¹¹⁷. For instance, palmitoylation has been shown to be essential for the stabilization of the N-terminal fragment (NTF) of tumor

necrosis factor α and, together with its partitioning inside lipid rafts, results in its cleavage by SPPL2b¹¹⁹.

From a mechanistic point of view, SPPL2a and SPPL2b appear to be very similar, since both enzymes cleave the same substrates with comparable efficiency when both substrate and protease are co-expressed¹⁰⁹. Although both SPPL2a and SPPL2b single knockout mice are viable and fertile, only SPPL2a exhibits a major immunological phenotype, characterized by a depletion of mature B cells, caused by a defect in CD74, under steady-state conditions^{109,120-122}. This difference is most likely dependent on the differential subcellular localizations of SPPL2a and SPPL2b, which, together with their distinct tissue-specific expression patterns, suggest non-redundant proteolytic functions of both proteases.

Emerging therapeutic roles of SPPL2 proteins in cardiovascular disease

To date, aside from associations, no functional role of SPPL2 proteins have been described in human disease. Previously, it was shown that absence of the SPPL2a protease results in a defect in B cell development and function^{109,120-122}, suggesting that SPPL2a might be used as a therapeutic target in B cell-dependent autoimmune disorders. Furthermore, we have shown that SPPL2a/b reduce pro-atherogenic LOX-1 signaling and their expression in the non-hematopoietic compartment protects against atherosclerosis development (**chapter 7**). However, the deleterious effects of LOX-1 are not only limited to atherosclerosis, but are also implicated in hypertension¹²³, arterial thrombosis¹²⁴, myocardial infarction¹²³ and renal dysfunction¹²⁵, suggesting that SPPL2a/b may also have a protective effect in these pathological conditions, although this remains to be determined.

Before SPPL2 proteins can be used as targets for therapeutic intervention, several issues have to be addressed. Firstly, before SPPL2 proteins should even be considered as therapeutic targets, their role in pathological conditions has to be defined. Furthermore, specific targeting methods have to be devised for each individual SPPL2 member. While increasing proteolytic activity is a feature difficult to achieve, except by overexpression, inhibiting enzyme function would be a more attractive option. However, currently available inhibitors lack specificity, since all members of SPPL2 subfamily and, in some cases, even the closely related presenilins are targeted¹²⁶. While there is high degree of sequence conservation between SPPL2a and SPPL2b, usage of currently available protease inhibitors indicate that the active site architecture differs between both proteins¹²⁶. Therefore, resolution of the crystal structure as well as identification of the substrate repertoire for each individual SPPL2 member, will aid in the development of specific inhibitors for individual SPPL2 proteins. In addition, it remains to be determined whether SPPL2 proteins harbor exosites that could be exploited similar to ADAM proteases.

Concluding remarks

In this thesis we addressed the immunomodulatory effects of HDL, and the effects of transmembrane proteolysis by the sheddases ADAM8 and ADAM10, and the I-CLiPs SPPL2a and SPPL2b on vascular inflammation. Although, HDL, ADAMs and SPPL2 proteases have arisen as attractive targets against atherosclerosis, there still remain several issues of fundamental importance that need to be addressed, including the complexity of HDL subpopulation heterogeneity, the cell-specific effects of HDL and ADAM10, the disease-specific effects of ADAM8 and ADAM10, and the obscurity of the SPPL2 proteins in health and disease, before embarking on a time-consuming and costly quest to treat CVDs using HDL-, ADAM- or SPPL2-based therapeutic strategies.

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Summary

Cardiovascular diseases (CVDs) are the leading cause of morbidity and mortality worldwide. A large proportion of CVD-related deaths are attributable to atherosclerosis, a lipid-driven chronic inflammatory disease. Statins have been the cornerstone in the treatment of atherosclerosis, however, despite their effectiveness in lowering LDL-cholesterol and preventing CVD events, there still remains a residual CVD risk that remains unresolved. Therefore, there is a need for new therapeutics that restore the lipid imbalance and/or resolve chronic inflammation in the atherosclerotic plaque. The inverse relationship between high-density lipoprotein (HDL) cholesterol and CVD risk, together with the role of HDL in the macrophage reverse cholesterol transport pathway as well as its immunomodulatory effects, has stimulated a great interest in therapeutically raising plasma HDL levels. In addition, post-translational proteolytic processing of transmembrane proteins, through ectodomain shedding by A Disintegrin And Metalloproteinases (ADAMs) or intramembrane proteolysis by signal peptide peptidase like 2 (SPPL2) proteases, has emerged as a novel level of control of many biological processes, including inflammation. Tight regulation of both processes is necessary to preserve homeostasis, however aberrant (de)activation may fuel pathological processes, including atherosclerosis development.

In this thesis, we explored the (immuno)modulatory capabilities of HDL and the membrane-associated proteases ADAMs and SPPL2 in macrophage-mediated immune responses and atherosclerosis development.

In **chapter 2**, we investigated the immunomodulatory effects of HDL in macrophages. Pre-incubation with HDL resulted in enhanced pro-inflammatory cytokine profile in murine and human primary macrophages *in vitro*, which were mediated by passive cholesterol depletion and lipid raft disruption. These effects were confirmed in peritoneal macrophages from apolipoprotein (apo)A-I transgenic mice, which have elevated levels of circulating HDL. Furthermore, macrophage dependent clearance of *P. aeruginosa* was compromised in apoA-I knockout mice, which have low levels of HDL, compared to wildtype and apoA-I transgenic mice. Genome-wide transcriptome profiling, together with a combinatorial pharmacological and genetic intervention approach, revealed that HDL enhances Toll-like receptor-induced signaling by activating a PKC-NF- κ B/STAT1-IRF1 axis. These findings indicate that HDL can support macrophage-mediated immune responses.

Given the inconsistent findings on HDL's immunomodulatory effects in macrophages, in **chapter 3**, we discussed the differences in methodologies, that potentially underlie the discrepancies, including the use of different macrophage types, concentrations or preparations of HDL, or readout models. Moreover, we provide evidence that the paradoxical findings are most likely not due to disease- or storage-associated structural modifications of HDL.

Chapter 4 summarized the current knowledge on the roles of ADAMs in cell migration in inflammation, wound healing and cancer metastasis. ADAMs may interfere with different steps of cell migration, either by proteolytic cleavage or by non-enzymatic activities.

In **chapter 5**, the causal involvement of ADAM8 in atherosclerosis was investigated. ADAM8 is shown to be upregulated in human advanced unstable atherosclerotic lesions, especially in foam cell-rich areas. Furthermore, macrophages deficient in ADAM8 have an attenuated cytokine secretion. However, despite these findings, both whole body as

well as hematopoietic ADAM8 deficiency did not affect advanced atherosclerotic lesion development.

Chapter 6 describes a role for endothelial ADAM10 in atherosclerosis development. Endothelial ADAM10 deficiency exacerbated atherosclerosis development in mice. In addition, atherosclerotic plaques from these mice contain intraplaque microvessels and hemorrhage, suggestive of major plaque destabilization. These findings provide evidence that endothelial ADAM10 is an atheroprotective factor.

In **chapter 7**, we showed that the pro-atherogenic lectin-like oxidized low-density lipoprotein receptor 1 (LOX-1) is subject to multiple proteolytic post-translational modifications. Ectodomain shedding by ADAM10 and endo-lysosomal degradation results in the production of membrane-bound N-terminal fragments (NTFs). We found that LOX-1 NTFs are substrates of the intramembrane proteases SPPL2a and SPPL2b. Pharmacological inhibition and genetic ablation of SPPL2a/b led to an accumulation of LOX-1 NTFs in the cell, which in turn could amplify LOX-1 pro-atherogenic signaling. Deletion of SPPL2/b in the non-hematopoietic compartment resulted in larger and more advanced atherosclerotic lesions in mice. Collectively, these findings identify intramembrane proteolysis by SPPL2a/b as a novel anti-atherogenic mechanism to suppress LOX-1-dependent signaling.

Finally, the main findings of this thesis, their implications as well as future perspectives in the use of HDL, ADAMs and SPPL2a/b as therapeutic targets are discussed in **chapter 8**.

Samenvatting

Hart- en vaatziekten zijn wereldwijd de belangrijkste oorzaak van morbiditeit en sterfte. Een groot aandeel van de hart- en vaatziekten-gerelateerde sterftegevallen is te wijten aan slagaderverkalking, een chronische ontstekingsziekte die aangedreven wordt door lipiden. Het gebruik van statines is de belangrijkste pijler in de behandeling van slagaderverkalking. Echter, ondanks de effectiviteit van statines in het verlagen van LDL-cholesterol (bekend als het slechte cholesterol) en het voorkomen van hart- en vaatziekten-gerelateerde sterftegevallen, blijft er nog steeds een restrisiko over dat nog niet is opgelost. Hierom is er een behoefte aan nieuwe therapieën die de balans in vetten in het bloed kunnen herstellen en/of de chronische ontsteking in atherosclerotische laesies kan opheffen.

De inverse relatie tussen high-density lipoprotein (HDL) cholesterol (bekend als het goede cholesterol) en het risico op hart- en vaatziekten, alsmede de rol van HDL in de macrofaag reverse cholesterol transport as en zijn regulerende effecten op ontsteking, hebben een grote interesse opgewekt in het therapeutisch verhogen van plasma HDL concentraties. Bovendien is het afknippen van transmembraan eiwitten door middel van ectodomain shedding en intramembrane proteolysis, bewerkstelligd door A Disintegrin And Metalloproteinases (ADAMs) en signal peptide peptidase like 2 (SPPL2) proteasen, respectievelijk, naar voren gekomen als een nieuwe manier om biologische processen, waaronder ontsteking, te reguleren. Een strakke regulatie van beide processen is noodzakelijk om de homeostase te behouden. Echter kan abnormale (de)activatie van deze processen pathologische processen, zoals slagaderverkalking, stimuleren.

In dit proefschrift hebben we de regulerende effecten HDL en de membraan-geassocieerde proteasen ADAMs en SPPL2 in macrofaag-gemedieerde ontstekingsreacties en slagaderverkalking onderzocht.

In **hoofdstuk 2** hebben we de modulaire effecten van HDL in macrofagen-gemedieerde ontstekingsreacties bestudeerd. Pre-incubatie van HDL resulteerde in een verhoogde uitscheiding van ontstekingsmediatoren in muizen en humane primaire macrofagen in vitro, welke gemedieerd waren door het passief onttrekken van cholesterol uit bepaalde locaties binnen het celmembraan, de zogenaamde vetilandsjes/lipid rafts. Deze effecten zijn bevestigd in macrofagen uit het buikvlies van apolipoproteïne (apo)A-I transgene muizen, welke verhoogde concentraties van HDL in hun bloedsomloop hebben. Verder was de opruiming van *P. aeruginosa* bacteriën, die afhankelijk is van een goed functionerend aangeboren immuunsysteem, verminderd in apoA-I deficiënte muizen, welke een lagere circulerende HDL concentratie hebben vergeleken met wildtype en apoA-I overproducerende muizen. Middels genetische profilering en een combinatie van farmacologische en genetische interventies, hebben we laten zien dat HDL Toll-like receptor geïnduceerde signaaltransductie verhoogt door activatie van de PKC-NF- κ B/STAT1-IRF1 as. Deze bevindingen tonen aan dat HDL macrofaag-gemedieerde ontstekingsreacties kan ondersteunen.

Gezien verschillende studies tegenovergestelde effecten van HDL in macrofagen laten zien, wordt in **hoofdstuk 3** de verschillen in methodiek, die deze discrepanties kunnen verklaren, bediscussieerd. Met name verschillen in het gebruik van verschillende macrofaag typen, in concentraties of bereidingswijze/samenstelling van het HDL en in uitleesmodellen, en niet zozeer ziekte- of opslag-geassocieerde structurele modificaties van HDL, liggen hieraan ten grondslag.

In **hoofdstuk 4** wordt een samenvatting gegeven van de huidige kennis van de rol van ADAMs in cel migratie in ontsteking, wondgenezing en metastasering (kanker). ADAMs

blijken verschillende stappen van de cel migratie te beïnvloeden zowel door proteolytische klieving als door niet-enzymatische activiteiten.

In **hoofdstuk 5** is de functionele betrokkenheid van ADAM8 in slagaderverkalking bestudeerd. We laten zien dat ADAM8 verhoogd tot expressie komt in humane geavanceerde instabiele atherosclerotische laesies, met name in schuimcel-rijke gebieden. Verder hebben ADAM8 deficiënte macrofagen een verlaagde uitscheiding van ontstekingsmediatoren. Echter, ondanks deze bevindingen, had ADAM8 deficiëntie in het gehele lichaam als in het witte bloedcel compartiment geen effect op de ontwikkeling van geavanceerde atherosclerotische laesies.

Hoofdstuk 6 beschrijft een rol voor endotheel cel ADAM10 in de ontwikkeling van slagaderverkalking. Deficiëntie van ADAM10 in endotheel cellen verergert de ontwikkeling van slagaderverkalking in muizen. Daarnaast bevatten atherosclerotische laesies van deze muizen intraplaque bloedvaatjes en bloedingen, welke een ernstige plaque destabilisatie suggereert. Deze bevindingen bewijzen dat endotheel ADAM10 een beschermende factor is tegen slagaderverkalking.

In **hoofdstuk 7** laten we zien dat de atherosclerose-bevorderende lectin-like oxidized low-density lipoprotein receptor 1 (LOX-1) onderworpen kan worden aan meerdere proteolytische klievingen. Afbraak door ADAM10 en andere enzymen resulteert in de productie van membraan-gebonden fragmenten. We hebben gevonden dat deze LOX-1 fragmenten substraten zijn van de intramembraan enzymen SPPL2a en SPPL2b. Farmacologische en genetische remming van SPPL2a/b leidde tot een ophoping van LOX-1 fragmenten in de cel, die op hun beurt de pro-atherosclerotische functie van LOX-1 konden versterken. Deletie van SPPL2a/b in vaatwand cellen resulteerde in grotere en meer geavanceerde atherosclerotische laesies in muizen. Tezamen identificeren deze bevindingen SPPL2a/b gemedieerde afbraak van LOX-1 aan het binnenmembraan van de cel als een nieuw LOX-1 activiteitsdempend en dus beschermend mechanisme in slagaderverkalking.

Ten slotte worden de belangrijkste bevindingen, hun implicaties en de toekomstperspectieven van het gebruik van HDL, ADAMs en SPPL2a/b als therapeutische targets bediscussieerd in **hoofdstuk 8**.

Valorization

Socioeconomic impact

Cardiovascular diseases (CVDs) are the leading cause of morbidity and mortality worldwide, accounting for ~31% of all global deaths¹. However, with the onset of metabolic disorders, including obesity and type 2 diabetes, the prevalence and incidence of CVDs is expected to rise. Currently, CVDs represent a major economic burden accounting for €210 billion a year in healthcare costs in Europe alone². Furthermore, as CVDs are often not instantly lethal, patients may require life-long treatment and suffer from severe disabilities. Moreover, CVDs may also indirectly have great impact for instance on people which are close to the afflicted patient.

Approximately 80% of all CVD-related deaths is attributable to coronary heart disease and stroke, which have atherosclerosis as underlying pathology¹. Atherosclerosis is a multifactorial lipid-driven chronic inflammatory disease of the medium- and large-sized arteries. Atherosclerosis develops during childhood and adolescence, and can remain clinically silent for decades. Risk factors include a family history of CVD, aging, hyperlipidemia, hypertension, diabetes and life-style factors such as stress, tobacco smoke, lack of physical activity and diets that are high in saturated and trans fatty acids, cholesterol, salt, and sugar. Currently available treatments involve surgical interventions or risk factor management through lifestyle changes and/or medications, such as 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, also commonly known as statins³. Statins have been the cornerstone in the treatment of atherosclerosis, however, despite their effectiveness in lowering LDL-cholesterol and preventing CVD events, there still remains a ~75% residual CVD risk that remains unresolved⁴. In addition, some patients are able to tolerate only low doses of statins, while other are unable to tolerate statin therapy at all⁵. Novel drugs, including lipid-lowering proprotein convertase subtilisin/kexin type 9⁶ and anti-inflammatory interleukin 1 β ⁷ inhibitors, show great promise in reducing CVD events on top of statin treatment, however they too are insufficient in fully preventing CVD-related deaths.

The socioeconomic burden caused by atherosclerosis raises demand for new innovative treatment strategies. Currently, the progression from an early fatty streak lesion to a rupture-prone atherosclerotic plaque is still incompletely understood. This implies that fundamental research is essential to improve our understanding of the atherosclerotic disease processes, which can yield new insights that will aid in the development of novel therapies for treating atherosclerosis, and thus prevent the majority of the CVD-related deaths.

High density lipoproteins

Raising high-density lipoprotein (HDL) cholesterol (HDL-C) has gained a lot of interest due to the inverse relationship between serum HDL-C levels and the risk of CVD events⁸⁻¹⁰. However, recent clinical trials using HDL-C raising agents, such as nicotinic acid, fibrates and cholesteryl ester transfer protein inhibitors, with the exception of anacetrapib, have failed to show any beneficial effect on CVD outcome¹¹⁻¹⁵. While anacetrapib doubled blood levels of HDL-C, the reduction in CVD risk on top of statin treatment of anacetrapib was mainly ascribed to its low-density lipoprotein cholesterol lowering effect¹⁵. Based on these observations, research has been refocused towards HDL particle functionality, more specifically its anti-atherogenic/-inflammatory functions, rather than its cholesterol content. A myriad of studies have shown that HDL exerts anti-inflammatory effects in endothelial cells (ECs) and vascular smooth muscle cells (VSMCs), two important vascular cell types which are intricately involved in the development and progression of atherosclerosis¹⁶. However, in contrast to the aforementioned cell types, we showed that

HDL exerts pro-inflammatory effects in macrophages (**chapter 2**), the main inflammatory cell type in atherosclerotic lesions ¹⁷. Therefore, HDL can either augment or inhibit the progression of a disease, depending on the cell type and cellular process it modulates.

In ECs, HDL exerts many anti-atherogenic effects, including the improvement of endothelial function and repair, as well as the attenuation of leukocyte recruitment by reducing the expression of adhesion molecules and chemokine ¹⁶. Moreover, infusion of reconstituted HDL increased capillary density and blood flow recovery in a murine ischemic hind-limb model of angiogenesis ¹⁸. However, due to its pro-angiogenic potential, HDL might also promote pathological neovascularization in atherosclerotic lesions, thereby promoting plaque instability, however, this remains to be determined.

In the case of VSMCs, HDL can inhibit their proliferation and thus limit neointimal hyperplasia ¹⁹. However, reduced VSMC proliferation in atherosclerotic lesions may cause plaque instability and rupture ²⁰. On the other hand, HDL also reduces chemokine secretion by VSMCs ¹⁹, which may limit leukocyte recruitment and atherosclerosis progression ²¹.

In macrophages, HDL activates pro-inflammatory signaling (**chapter 2**), which has been associated with atherosclerotic plaque instability ²². Moreover, we also found that HDL enhanced bacterial phagocytosis (**chapter 2**), a process which shows a high degree of overlap with efferocytosis, the phagocytosis of apoptotic cells ^{23,24}. However, in contrast to bacterial phagocytosis, efferocytosis dampens the inflammatory response by removing immunogenic debris and stimulating the production of anti-inflammatory mediators ²¹. Moreover, we have shown that HDL can aid in the clearance of bacterial infections. Interestingly, bacterial infections have been shown to accelerate atherosclerosis development in mice ^{25,26}. Therefore, HDL may limit the exacerbation of atherosclerosis induced by bacteria.

Collectively, HDL can exert both pro- and anti-atherogenic effects in the aforementioned cell types and it will be the net effect of HDL on the investigated cell types that will dictate the course of the disease.

Studies in preclinical animal models, especially mice, show that raising plasma HDL levels can inhibit or even reverse both early and advanced atherosclerosis development. Furthermore, infusion of reconstituted HDL reduces plaque volume size in humans ²⁷, suggesting that the net effect of HDL is anti-atherogenic. However, all (pre)clinical studies on HDL to date have relied on surrogate markers for atherosclerotic plaque stability, including lesion size/volume and lipid-, macrophage- and collagen content, rather than on clinical manifestations, such as myocardial infarction or stroke. Therefore, the pro-inflammatory effects of HDL in macrophages might still be relevant in terms of plaque rupture and subsequent atherothrombotic events. Reconstituted HDL CER-001 and CSL-112, which were developed by Cerenis Therapeutics and CSL Behring, respectively, are currently used in clinical trials as a treatment for coronary heart disease. The results of these studies will provide evidence whether raising plasma HDL levels is a viable therapeutic strategy against CVDs and whether the pro-inflammatory effects of HDL in macrophages are clinically relevant in the management of CVD by HDL raising therapies. In addition, based on our findings, HDL therapy may be useful in patients with persistent or recurrent bacterial infections.

Transmembrane proteases

In contrast to HDL, the use of A Disintegrin And Metalloproteinases (ADAM)8 and 10 or signal peptide-peptidase-like (SPPL) 2a and 2b as therapeutic targets is still far removed from clinical application. First of all, under homeostatic conditions, protease activity is delicately balanced to accommodate the needs of the cell or tissue. However, disturbances in proteolytic activity through a dysregulation in transcriptional control, alternative splicing, post-translational modifications, subcellular trafficking and localization, protein-protein interactions, the presence/absence of natural inhibitors, and in the abundance of both protease and substrate can have dramatic effects resulting in the development and/or progression of a disease ²⁸. Second, the design of protease specific inhibitors appears to be rather challenging, because they are designed to block the active site of an enzyme, which contains residues that are often well conserved in a family of proteases, as is the case with members of the metalloprotease family, like ADAMs and matrix metalloproteases. Third, most if not all proteases have more than one substrate rendering them capable of affecting multiple pathways, however the net result seems to depend on the cell type and context. Previously, we have shown that myeloid ADAM10 promotes atherosclerotic plaque instability by stimulating a pro-degradative/inflammatory phenotype in macrophages ²⁹. In contrast, endothelial ADAM10 is atheroprotective by limiting pathological intraplaque neovascularization and hemorrhage (**chapter 6**). Furthermore, ADAM10 can enhance the migration and invasiveness of cancer cells thereby promoting cancer metastasis ³⁰. Collectively, targeting ADAM10 will warrant a cell type- and disease-specific approach. For atherosclerosis, this will entail the inhibition of ADAM10 activity specifically in atherosclerotic plaque macrophages through targeted delivery of ADAM10 enzyme inhibitors or antisense oligonucleotides, using for example ligand-targeted nanoparticles as a delivery system ³¹. This has to be accomplished without affecting macrophages in other tissues, since reducing the pro-inflammatory response of macrophages will hamper their response to micro-organisms and their antitumor activity for example ³². Furthermore, using a similar strategy as for plaque macrophages, reducing ADAM10 activity in cancer cells will limit their metastatic potential. On the other hand, increasing ADAM10 activity specifically in endothelial cells may limit atherosclerosis development, however, to date, there is no therapeutic that can increase the proteolytic activity of a specific protease. While overexpression of a protease can increase the abundance of a protein, it does not mean that the protein is proteolytically active.

In contrast to ADAM10, ADAM8 is upregulated in many inflammatory diseases and is not required under homeostatic conditions in adulthood, at least in mice ³³⁻³⁷. This makes ADAM8 an excellent target for intervention. Indeed, inhibiting ADAM8 function attenuates bronchial hyperresponsiveness and inflammation in murine asthmatic models ³⁸, as well as pancreatic tumor burden and invasiveness in mice ³⁹. However, we show that ADAM8 is not involved in atherosclerosis development (**chapter 5**), thus ADAM8 is not a viable target for intervention in the treatment of atherosclerosis.

Unlike ADAM proteases, research on SPPL2 proteases is still in its infancy. We have shown that SPPL2a/b limit pro-atherogenic LOX-1 signaling and their expression in the non-hematopoietic compartment protects against atherosclerosis development (**chapter 7**). Interestingly, deletion of SPPL2a alone results in an overt immunological phenotype, characterized by depletion of mature B cells, suggesting that SPPL2a might be a viable therapeutic target in B cell-dependent autoimmune diseases ⁴⁰. In addition, B cell depletion was shown to reduce atherosclerosis development ⁴¹, suggesting that SPPL2a deletion in the hematopoietic, which also includes leukocytes, may limit atherogenesis, opposite to its effect in the non-hematopoietic compartment. However, this requires

further investigation. Given the fact that LOX-1 is also implicated in many other diseases⁴²⁻⁴⁴ and the full substrate repertoire of SPPL2a/b is still unresolved⁴⁰, it remains to be determined whether targeting SPPL2a/b can be beneficial in other diseases besides atherosclerosis.

Conclusion

In conclusion, while raising plasma HDL levels is a promising therapeutic strategy in the treatment of atherosclerosis, caution is warranted in its implementation in the clinic due to potential adverse effects in macrophages. Furthermore, although ADAM8 is not a viable target for intervention, ADAM10 requires a cell type- and disease-specific approach for it to be exploited as a therapeutic target against atherosclerosis. Lastly, targeting SPPL2a/b in atherosclerosis requires further investigation before it can be regarded as a suitable target for intervention.

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List of abbreviations

ABCA1	ATP-binding cassette subfamily A1
ABCG1	ATP-binding cassette subfamily G1
ADAM	A Disintegrin And Metalloproteinase
apoA-I	apolipoprotein A-I
apoB	apolipoprotein B
aSMA	α smooth muscle actin
BAL	Bronchoalveolar lavage
BMDM	Bone marrow-derived macrophage
BMT	Bone marrow transplantation
CETP	Cholesteryl ester transfer protein
cHDL	commercial high density lipoprotein
CVD	Cardiovascular disease
DC	Dendritic cell
EC	Endothelial cell
ECM	Extracellular matrix
EL	Endothelial lipase
ERK	Extracellular signal-regulated kinase
HCD	High cholesterol diet
HDL	High density lipoprotein
HL	Hepatic lipase
HSC	Hematopoietic stem cells
ICAM-1	Intracellular adhesion molecule 1
ICD	Intracellular domain
I-CLiP	Intramembrane cleaving protease
IL	Interleukin
iMAEC	immortalized murine aortic endothelial cell
IPH	Intraplaque hemorrhage
LCAT	Lecithin-cholesterol acyltransferase
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
LOX-1	Lectin-like oxidized low-density lipoprotein receptor 1
LPS	Lipopolysaccharide
MAPK	mitogen-activated protein kinase
MEF	murine embryonic fibroblast
MMP	Matrix metalloproteinase
M β CD	Methyl- β -cyclodextrin
nHDL	native high density lipoprotein
NO	Nitric oxide
NTF	N-terminal fragment
oxLDL	Oxidized low density lipoprotein
PC	Phosphatidylcholine
PCSK9	Proprotein convertase subtilisin/kexin type 9
PKC	Protein kinase C
PM	Peritoneal macrophage
pMAC	primary mouse aortic cells

PSGL-1	P-selectin glycoprotein-1
RCT	Reverse cholesterol transport
rHDL	reconstituted high density lipoprotein
RIP	Regulated intramembrane proteolysis
sLOX-1	soluble lectin-like oxidized low-density lipoprotein receptor 1
SNP	Single nucleotide polymorphism
sPLA2	Secretory phospholipase A2
SPPL	Signal peptide peptidase-like
SR-B1	Scavenger receptor B1
STAT1	Signal transducer and activator of transcription 1
TG	Triglyceride
TIMP	Tissue inhibitor of metalloproteinase
TLR	Toll-like receptor
TNF α	Tumor necrosis factor α
VCAM-1	Vascular cell adhesion molecule 1
VE-Cadherin	Vascular endothelial cadherin
VLDL	Very low density lipoprotein
VSMC	Vascular smooth muscle cell

Curriculum vitae

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Theodorou K*, van der Vorst EP*, Gijbels MJ, Wolfs IMJ, Jeurissen M et al. Whole body and hematopoietic ADAM8 deficiency does not influence advanced atherosclerotic lesion development, despite its association with human plaque progression.

**equal contribution*

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Theodorou K*, van der Vorst E.P.C*, Rademakers T, Theelen T.L, Sluimer J.C, Wijnands E et al. Endothelial ADAM10 deficiency augments murine atherosclerosis and is associated with intraplaque neovascularization and hemorrhage. **equal contribution*
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04/2017	Paper of the year award Scandinavian Society for Atherosclerosis Research, Humlebaek, Denmark
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hartelijk bedankt

many thanks

dankeschön

mercikes

merci beaucoup

çox sağ olun

ευχαριστώ πολύ

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