Innate immune modulation in atherosclerosis and vascular

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Chapter 1

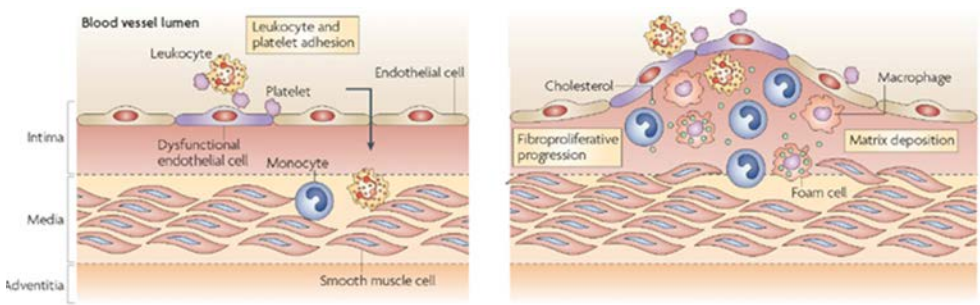
General Introduction

Atherosclerosis

Atherosclerosis is a chronic, multifactorial disease in which multiple inflammatory processes and modifications in cholesterol homeostasis play a key role. The formation of atherosclerotic lesions in the innermost layer of the vessel wall, the intima, occurs at predisposed sites in the arterial tree located near branch points and inner curvatures[1]. These asymmetric, focal thickenings in the vessel wall consist of lipids, debris and various inflammatory cells. Rupture of an advanced atherosclerotic plaque can result in thrombus formation, which may occlude the blood vessel and lead to severe cardiovascular complications. In fact, atherosclerosis is the major cause of cardiovascular diseases (CVD) such as peripheral ischemia, myocardial infarction and stroke[2]. Pre-emptive therapeutic options include the use of statins and antihypertensive drugs, also, lifestyle changes such as smoking cessation are recommended, which reflects the multifactorial nature of the disease[3]. Despite an improved treatment, CVD are still responsible for 1 in every 4 deaths in the Netherlands[4]. It is therefore important to identify new therapeutic targets to prevent the initiation, growth and rupture of an atherosclerotic plaque.

Early atherosclerosis

Already early in life, around the age of 20 to 30, the first signs of atherosclerosis are present in the vessel wall, without any clinical manifestations yet[5]. The initiation of these early atherosclerotic plaques, also referred to as 'fatty streaks', starts at sites of damaged or activated endothelium. Activated endothelial cells up-regulate the expression of several adhesion molecules, such as ICAM-1, VCAM-1 and selectins, thereby causing rolling and subsequent adherence of monocytes and other leukocyte subtypes[6]. These leukocytes are subsequently stimulated to migrate through the endothelial junctions by combined actions of PECAM-1 and various chemokines produced in the intima. Once present in the intima, monocytes differentiate into macrophages, which then take up lipids via their scavenger

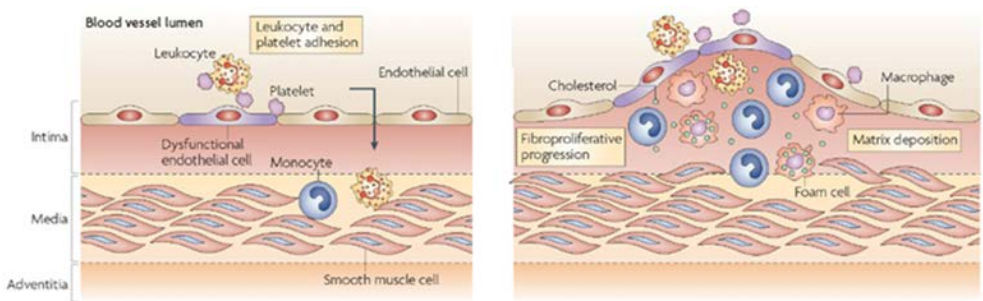


During the initiation of atherosclerosis, leukocytes adhere to the activated endothelium and subsequently migrate into the intima (left panel). Gradual intimal thickening caused by the accumulation of lipid-laden macrophages (foam cells) leads to the formation of fatty streaks (right panel). Adapted and modified from Weber et al. Nat Rev Immunol 2008;8:802-15.

receptors and transform into foam cells. Early fatty streaks may disappear again in time or eventually progress to advanced atherosclerotic lesions[7].

Advanced atherosclerosis

Growth of a plaque is caused by the continued influx and activation of inflammatory cells such as monocytes, T cells and mast cells. Several endogenous ligands in the lesion, such as oxidized LDL and damage associated molecular patterns (DAMPs) are able to activate immune cells via pattern recognition receptors such as Toll-like receptors (TLRs), causing them to release various cytokines and chemokines thereby amplifying the inflammatory process[8]. In addition, secreted cytokines and growth factors induce a phenotypic switch in smooth muscle cells from the media, from a resting contractile state into a proliferative state[9,10]. These smooth muscle cells then migrate into the intima where they produce collagen and other extracellular matrix components, which results in the formation of a fibrous cap. Inflammation, hypoxia and excessive protease activity in the plaque can promote cellular apoptosis, leading to the formation of a necrotic core underneath the fibrous cap. These advanced atherosclerotic plaques do not necessarily lead to clinical events, as lesions containing a thick fibrous cap and preserved lumen area may remain stable for years without apparent clinical manifestations[11].



Impaired clearance of apoptotic cells results in the formation of a necrotic core, which is covered by a fibrous cap. Multiple immune cells and leaky neovessels present in the plaque contribute to the inflammation (left panel). Rupture of the fibrous cap leads to exposure of the necrotic core to the blood, resulting in thrombus formation (right panel). Adapted and modified from Weber *et al*. Nat Rev Immunol 2008;8:802-15.

Atherosclerotic plaque destabilization

Unstable atherosclerotic lesions have been extensively described based on histology and plaque morphology[11,12]. A typical unstable lesion consists of a large necrotic core, covered by a thin fibrous cap. A high density of leaky neovessels derived from the adventitial *vasa vasorum* contributes to increased leukocyte infiltration. The inflammatory cell content is high, in particular at the rupture-prone shoulder regions of the plaque[13]. Thinning of the fibrous cap is the result of smooth muscle cell apoptosis, which may be caused by for example chymase derived from the mast cell[14] or by complement component C5a[15]. Also, matrix

metalloproteinases (MMPs) impair the integrity of the fibrous cap due to increased breakdown of the collagen content. Eventually, rupture (or erosion) of the cap may occur, leading to exposure of the pro-thrombotic necrotic core to the blood flow[16]. Subsequent thrombus formation can result in either distal embolization or in incorporated lesional thrombi, thereby adding to the plaque burden[17].

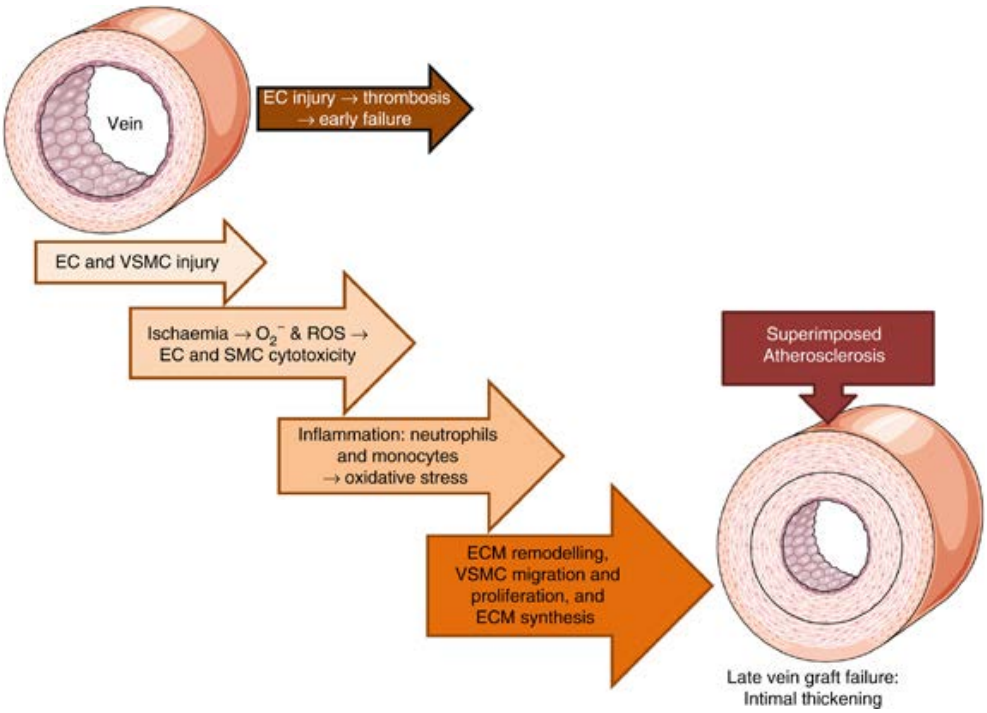
Vascular intervention techniques

Various surgical techniques, such as percutaneous transluminal angioplasty (PTA), stenting, atherectomy and placement of bypass conduits have been developed in order to treat or prevent atherosclerotic complications. During PTA, a balloon is inserted with the use of a catheter into the blood vessel and inflated at the site of the atherosclerotic lesion. The plaque is then compressed into the vessel wall, thereby widening the lumen and increasing blood flow. PTA is often combined with stenting, in which a small metal tube is placed at the site of the narrowed artery in order to maintain the appropriate lumen area. During atherectomy a catheter with a sharp blade is utilized to discard the atherosclerotic plaque from the arteries, this may also be done in an open surgical procedure referred to as endarterectomy. Finally, bypassing the blocked artery with either arterial or venous conduits is also a commonly used surgical treatment for atherosclerosis. Unfortunately, all of the above-mentioned techniques have their drawbacks, severely impairing the success rate of these operations. Damage to the vessel wall induced by atherectomy, balloon angioplasty or stenting generates a local inflammatory reaction, which results in restenosis, caused by accelerated neointimal formation. Also, excessive intimal hyperplasia occurs in bypass segments, which may lead to failure of the graft. Still, venous bypass grafts are commonly used due to their easy accessibility and the advantage of their long length, making it possible to bypass multiple atherosclerotic lesions[18].

Vein graft disease

Vein graft disease (VGD) can be divided in early graft failure, due to thrombosis or technical complications, or in midterm to late failure, caused by atherosclerosis and intimal hyperplasia[19]. After one year, already 15-20% of the venous bypass grafts fail, which is increased up to 60% after ten years[20–22]. Placement of a vein in the arterial circulation, with a corresponding high blood pressure, results in immediate endothelial damage of the vein graft. This is followed by fibrin depositions, platelet adhesion and leukocyte transmigration into the vessel wall in the first week after the operation[23]. Smooth muscle cells from the media then

start to proliferate and migrate into the intima, which is necessary for the strength of the vein graft. However, excessive smooth muscle cell proliferation contributes to typical hyperplasia of the vessel wall. Complete occlusion of the graft may eventually be caused by intimal hyperplasia, or because rupture of the vein graft lesion results in thrombosis[24].



Following endothelial cell damage after vein graft surgery, immediate failure of the graft may be caused by occlusive thrombosis. Late graft failure may be the result of excessive smooth muscle cell proliferation, inflammation and extracellular matrix deposition, leading to decreased blood flow due to intimal thickening. Adapted from Wan *et al*. Gene Ther. 2012;19:630-6.

Murine models for atherosclerosis and vein graft disease

In order to study the mechanisms behind the complex, multifactorial nature of atherosclerosis and vein graft disease, the use of animal models is indispensable. Mice are the most commonly used animals for these experiments due to various advantages, such as their short breeding time and their well-known genetic background. Also, the execution of transgenic studies and specific gene targeting is possible in murine models. However, mice are highly resistant to atherosclerosis, even when they are fed a high cholesterol diet[25]. Therefore, apoE knockout (apoE$^{-/-}$) mice have been generated by inactivating the apolipoprotein E gene, which results in high VLDL and LDL plasma levels with early atherosclerotic lesion formation in the aortic root. These mice will develop advanced atherosclerotic

plaques[26] when placed on a “western type diet” containing fat and cholesterol. In contrast to the apoE$^{-/-}$ mice, LDL receptor deficient mice (LDLr$^{-/-}$) do not develop atherosclerosis under basal conditions. However, LDLr$^{-/-}$ mice are very responsive to high cholesterol diet feeding, resulting in the formation of atherosclerotic lesions when fed for example a western type diet[27,28].

Collar model

Rapid, site-specific atherosclerotic lesions can be induced by placing perivascular collars in apoE$^{-/-}$ or LDLr$^{-/-}$ mice on a western type diet. In this experimental setup, nonconstrictive collars prepared from silastic tubes are placed around both common carotid arteries. Disturbed flow at the proximal site of the collar coincides with a downregulation of Krüppel-like factor 2 (KLF2), an endothelial transcription factor regulated by shear stress[29], while the adhesion molecules ICAM-1 and VCAM-1 are strongly upregulated[25]. These hemodynamic changes, combined with a western type diet, result in advanced atherosclerotic lesions after 4 weeks.

Vein graft model

The most widely used experimental model for vein graft disease is introduced by Xu[30] over 15 years ago. In this model, the vena cava from a donor mouse is interplaced in the carotid artery of the recipient mouse. After harvesting the caval vein, the carotid artery of the recipient mouse is dissected free, ligated, and cut in the middle. The ends of the arteries are inversed around two cuffs, after which they are fixed with a ligature. Finally, the donor caval vein is sleeved over both cuffs and is fixated with two ligatures. A visible pulsatile flow confirms successful engraftment. Immediate endothelial damage of the vein graft due to the high blood pressure in the arterial circulation results in vessel wall thickening as early as 1 week after surgery. After 4 weeks, the intimal hyperplasia is even progressed to a 10-fold increase compared to the original thickness. Vein graft surgery in hypercholesterolemic mice results in an even more aggravated lesion formation with atherosclerotic features such as foam cell accumulation.

Interestingly, lesions induced in a murine vein graft model display a complex morphology including neovascularization, calcifications and disruptions consisting of fibrin depositions and intraplaque hemorrhage[31]. These disruptions are not completely comparable to plaque ruptures in the classical definition, in which a thin cap covering a lipid core ruptures, resulting in atherothrombosis[16]. However, investigating plaque rupture in mouse models has always been challenging since there are no mouse models available in which plaque ruptures occur in a similar manner as in the human situation. Therefore, the vein graft model is, besides an interesting model to investigate novel therapeutic strategies in vein graft disease, also a useful tool to study underlying mechanisms of plaque disruptions.

The immune system

Traditionally, the immune system has been divided into innate and adaptive immune responses, both of which play an important role in atherosclerosis. Monocytes, macrophages, dendritic cells, neutrophils, mast cells and NK cells all belong to the innate immune system and act as a first line of defense against common microorganisms and infections. These cells make use of germ line encoded pattern recognition receptors for pathogens or damaged self components, such as TLRs and scavenger receptors[32]. Pathogens are internalized, degraded and subsequently presented by antigen presenting cells (APCs) such as dendritic cells, which initiates the adaptive immune response. Adaptive immunity comprises clonally diverse lymphocytes with unique antigen recognition receptors, which allows them to recognize almost every possible pathogen en subsequently develop an antigen-specific immune response[33]. Activation of the T and B lymphocytes results in cytokine secretion, antibody production and in memory formation, the latter being a typical feature of the adaptive immune system.

Monocytes

Recruitment of monocytes to the site of inflammation is a key mechanism in the initiation of atherosclerosis[34]. Two distinct monocyte subsets in the blood have been described: classical monocytes ($CD14^{+}CD16^{-}$ in human; $Ly6C^{+}CX3CR1^{low}CCR2^{+}$ in mice) and non-classical monocytes ($CD14^{low}CD16^{+}$ in human; $Ly6C^{-}CX3CR1^{high}CCR2^{-}$ in mice)[35]. Non-classical monocytes constantly patrol healthy tissue by transient crawling along the endothelium, which allows rapid tissue invasion in case of infection or inflammation[36]. Following this early response, classical monocytes are primarily recruited to the site of inflamed tissue, where they produce high levels of inflammatory cytokines[37]. Interestingly, these classical $Ly6C^{high}$ monocytes are markedly increased in hypercholesterolemic $apoE^{-/-}$ mice, where they adhere to activated endothelium and infiltrate into atherosclerotic lesions[38]. It has previously been shown that suppression of monocyte recruitment in atherogenesis reduces plaque size, however, similar effects were not observed in late stage atherosclerosis, which indicates the importance of monocyte influx in early atherogenesis[39].

Different chemokine receptors, amongst which are CCR1, CCR2, CCR5 and CX3CR1, are involved in the influx of monocytes to the atherosclerotic lesion. For instance, several groups have shown that deficiency or inhibition of CCR2 markedly decreases plaque size[40–42], stressing the importance of chemokine-receptor signalling pathways in monocyte recruitment. Once infiltrated into the vessel wall, monocytes differentiate into lesional macrophages, which engulf modified LDL, transform into foam cells, and contribute to the ongoing inflammatory process through the excretion of inflammatory mediators.

Macrophages

Macrophages are a heterogenic population of cells with diverse roles in development, homeostasis, tissue repair and immunity. They have the ability to efficiently phagocytose and kill pathogens as well as remove cellular debris in inflamed tissues. Moreover, they can present processed peptides to T cells thereby assisting in the execution of an immunological response[43].

The accumulation of macrophages and lipids underneath the endothelial cell layer is the initial hallmark of the atherosclerotic process, leading to the formation of early 'fatty streaks'. These macrophages engulf modified lipids via phagocytosis or via scavenger receptors, such as CD36 or scavenger receptor A1 (SR-A1), which are then digested in the lysosome[44]. The resulting free cholesterol may be effluxed from the cell or it may be esterified and stored in cytosolic lipid droplets. Excessive free cholesterol accumulation increases pro-inflammatory signalling, leading to the activation of nuclear factor-κB (NFκB) with subsequent production of proinflammatory cytokines. Translocation of NFκB to the nucleus may also be induced via TLR mediated activation by ligands such as oxidized LDL or DAMPs[45]. However, not all the macrophages in the atherosclerotic plaque are thought to increase the inflammatory status of the lesion. Initially, a model was proposed in which macrophages were suggested to be of either a pro-inflammatory phenotype (M1, classically activated macrophage), or a wound-healing, anti-inflammatory phenotype (M2, alternatively activated macrophage)[46]. LPS, IFNγ and IL-1β are capable to induce M1 macrophages while the peroxisome proliferator-activated receptor-γ (PPARγ) and the Th2 related cytokines IL-4 and IL-13 are able to polarize macrophages towards an M2 phenotype[47]. M1 macrophages may aggravate atherosclerosis by excreting large amounts of inflammatory mediators such as IL-1β, IL-6, IL-8 and TNFα. Also, the production of reactive oxygen species (ROS) leads to oxidative stress, and various M1 derived MMPs (MMP1, MMP3, MMP9) may contribute to thinning of the fibrous cap by collagen degradation. M2 macrophages on the other hand are thought to resolve the inflammation via secretion of anti-inflammatory cytokines such as IL-10[48]. Since the initial division of macrophages into M1 and M2, other subsets such as Mox or M4 macrophages have been recognized as well, reflecting the highly heterogeneous nature of macrophages[49,50]. The exact number of macrophage subsets with their corresponding functions is currently still under debate, and it is likely that additional classifications will ensue in the future.

In late stage atherosclerosis, clearance of apoptotic cell debris and lipids by macrophages is impaired, leading to the formation of a necrotic core. A large necrotic core accompanied by a high number of inflammatory macrophages is an important feature of unstable lesions, prone to rupture[13]. Therapeutic approaches have focused on shifting macrophages from an inflammatory M1 to an anti-

inflammatory M2 phenotype, on cholesterol efflux mechanisms and on egress of macrophages from the atherosclerotic lesion.

Mast cells

Mast cells are effector immune cells of the innate immune system capable of excreting a broad spectrum of proteases, chemokines, cytokines and growth factors. These potent inflammatory cells are derived from bone marrow precursor cells and differentiate into mature mast cells upon migration into the tissue where they reside, under the influence of stem cell factor and IL-3[51]. Typically, mast cells are present in various mucosal tissues and in the skin; in close proximity to blood vessels, lymphatics and nerves[52]. The typical morphology of the mast cell, caused by the vast amount of granules they contain, led to their name 'Mastzellen', which means well-fed cells[53]. Mast cell granules are loaded with a vast amount of inflammatory preformed mediators, such as the mast cell specific tryptases, chymases and carboxypeptidase A3 (CPA3) inflammatory cytokines (e.g. TNFα) and histamine[54]. Upon activation, these secretory granules are acutely released into the extracellular environment. Various stimuli are capable to induce mast cell activation, amongst which are IgE and IgG immune complexes; the complement components C5a[15,55] and C3a[56] and the neuropeptides substance P[57] and NPY[58]. Also, mast cells express different TLRs via which they may be activated, however, this route of activation does generally not cause degranulation but instead only induces the release of cytokines and chemokines[59].

Although it is already 60 years ago that the mast cell has been described in relation to atherosclerosis[60], it is not until the past 15 years that its importance receives increasing recognition. Interestingly, high numbers of activated mast cells are detected in the rupture-prone shoulder regions of human atherosclerotic plaques[61]. An increased amount of mast cells in the plaque of patients suffering from carotid artery stenosis is even found to correlate with intraplaque hemorrhage and the occurrence of future cardiovascular events[62]. Moreover, a causal role is established linking mast cell activation to both atherosclerotic plaque growth and destabilization. Systemic mast cell activation in apoE$^{-/-}$ mice with a DNP hapten results in an increased lesion size in the brachiocephalic artery, while mast cell inhibition with the general stabilizer cromolyn significantly reduces this effect[63]. One mode of action via which the mast cell is thought to aggravate atherosclerosis is via the potent proteases chymase and tryptase. Inhibition of chymase does not only reduce lesion size, plaque stability is also increased as measured by a decreased necrotic core area, increased collagen content and a reduced number of intraplaque hemorrhages[64]. Moreover, *in vitro* and *in vivo* evidence indicates that mast cell chymase can induce smooth muscle cell apoptosis, leading to plaque destabilization[14,65]. The importance of mast cell derived tryptase has been shown

after lentiviral overexpression of tryptase in apoE$^{-/-}$ mice, which increases both atherosclerotic plaque size and intraplaque hemorrhages[66]. Mast cell derived inflammatory cytokines are major contributors to the atherosclerotic process as well, in particular IL-6 and IFNγ. This was investigated by the adoptive transfer of IL-6, IFNγ and TNFα deficient mast cells into mast cell deficient LDLr$^{-/-}$/Kit$^{W\text{-}sh/W\text{-}sh}$ mice. Interestingly, the reduced lesion size observed in these mice was again increased after the transfer of TNFα deficient mast cells, but not after that of IL-6 or IFNγ deficient mast cells, indicating an important role for these cytokines in atherogenesis[67]. Furthermore, the potent angiogenic mediator basic fibroblast growth factor (bFGF) is stored and secreted by the mast cell. A positive correlation is found between numbers of bFGF positive mast cells and microvessels in the plaque, which is increased with the severity of atherosclerosis[68]. Thus, there are a number of ways via which the mast cell can contribute to the growth and destabilization of atherosclerotic plaques.

Mast cell activation in the context of atherosclerosis may be induced by various ligands. For instance, the bioactive lysophospholipid acid (LPA) is present in atherosclerotic plaques, and local administration of LPA in apoE$^{-/-}$ mice results in increased mast cell activation and plaque destabilization[69]. Other mast cell ligands present in the atherosclerotic plaque include C5a, substance P, neuropeptide Y, and oxidized LDL, which may all contribute to mast cell activation, with subsequent aggravation of atherosclerosis[55,57,58,70].

Neutrophils

Neutrophils, the most abundant white blood cells in the circulation, are terminally differentiated cells derived from the bone marrow[71]. They are relatively short-lived cells which continuously patrol the blood in search for pathogens and apoptotic cells. Neutrophils can be recruited to the site of inflammation by various chemotactic agents, such as fMLP, C3a, C5a and a number of chemokines (e.g. IL-8)[72]. After transmigration through the endothelium, neutrophils may become activated and release their preformed granules which contain vast amounts of reactive oxygen intermediates, myeloperoxidase (MPO), cytokines, chemokines and the matrix degrading proteases MMP2 and MMP9[73]. Also, it has been recently described that neutrophils may expel their DNA in order to trap bacteria, in a process called NETosis (neutrophil extracellular traps)[74,75].

The role of neutrophils in atherosclerosis has not been extensively studied yet, which may be caused by their rare detection in the lesions due to their short lifespan or because of their ability to undergo phenotypic changes, resulting in the expression of markers normally present on antigen-presenting cells[76]. However, some studies have established a role for neutrophils in different stages of atherosclerosis. Neutrophils are detected in early and advanced lesions, in

particular in rupture-prone shoulder lesions[77]. Also, circulating neutrophils are increased in apoE$^{-/-}$ mice fed a high cholesterol diet, which is directly correlated to an increase in early atherosclerotic lesion size[78]. Moreover, neutrophils are present in human plaques and an increase in their numbers has been shown to correlate with unstable lesions[79].

Dendritic cells

Dendritic cells (DCs), named for their dendrite-like projections, are professional antigen presenting cells derived from hematopoietic precursors in the bone marrow. They link the adaptive and innate immune system and have the potential to either stimulate or suppress inflammatory responses[80]. Immature dendritic cells (iDCs) patrol the peripheral blood and tissues in search for antigens, which they can efficiently take up and process intracellularly. Uptake of antigens in the presence of "danger" signals, such as LPS or inflammatory cytokines, leads to the maturation of DCs[81]. This is accompanied by an upregulation of antigen-presenting molecules and co-stimulatory molecules on their cell surface, while the phagocytic activities are downregulated[82]. The processed antigens are loaded on the cell surface in the context of major histocompatibility (MHC) class I and II; subsequently the DCs will migrate to draining lymph nodes and present these antigens to naïve and memory T cells[83]. Antigen presentation in combination with the presence of co-stimulatory molecules induces a profound immune response via the activation and clonal expansion of T cells. On the other hand, tolerogenic DCs may suppress inflammation via the induction of regulatory T cells (Treg)[84].
In atherosclerosis, DCs have been detected in the vicinity of neovessels as well as near vasa vasorum in the adventitia, in close proximity to T cells[85]. DCs are involved in both early and late stages of atherosclerosis and have been described to be both detrimental and protective in atherosclerosis. While some groups have reported that CD11c deficiency in mice fed a high cholesterol diet attenuates atherosclerosis[86,87], others did not observe any effect on plaque size[88]. Deletion of non-classical DCs in LDLr$^{-/-}$ mice results in exacerbated atherosclerotic lesion formation with reduced numbers of Tregs, indicating a protective role of non-classical DCs in atherosclerosis[89]. Moreover, a beneficial contribution of DCs has been described after the transfer of oxLDL-pulsed mature DCs into LDLr$^{-/-}$ mice, which markedly reduces lesion size[90].

T cells

T cells are derived from hematopoietic stem cells in the bone marrow; however, they undergo their development in the thymus. After exiting the thymus, naïve T cells travel to secondary lymphoid organs to survey for antigens for which they are specific. In these secondary lymphoid organs DCs present processed

antigens on MHC class I, recognized by $CD8^+$ T cells (cytotoxic T cells), or on MHC class II, recognized by $CD4^+$ T cells (T helper cells)[91]. Activation of the T cell further requires a co-stimulatory signal from the APCs as well as the presence of cytokines. Subsequently, $CD4^+$ T cells acquire effector functions, which results in the formation of different subsets depending on the cytokine environment. The classical T helper 1 (Th1) cells are induced by IL-12 and IFNγ, while IL-2 and IL-4 are critical for Th2 differentiation. Development of Th17 cells is mostly initiated by IL-6, TGFβ and the master regulator RORγt[92]. Another subset comprises regulatory T cells (Tregs), which require the transcription factor Foxp3 for their development and function. Tregs have immunosuppressive functions through secretion of the inhibitory cytokines IL-10 and TGFβ[93]. Activation of naïve $CD8^+$ T cells results in the differentiation of cytotoxic T lymphocytes (CTLs), which may induce apoptosis of target cells (through granzymes and perforin) and promote inflammation via secretion of IFNγ and TNFα[94]. Following differentiation, effector T cells migrate to non-lymphoid tissues for a second activation by APCs that present the same antigen.

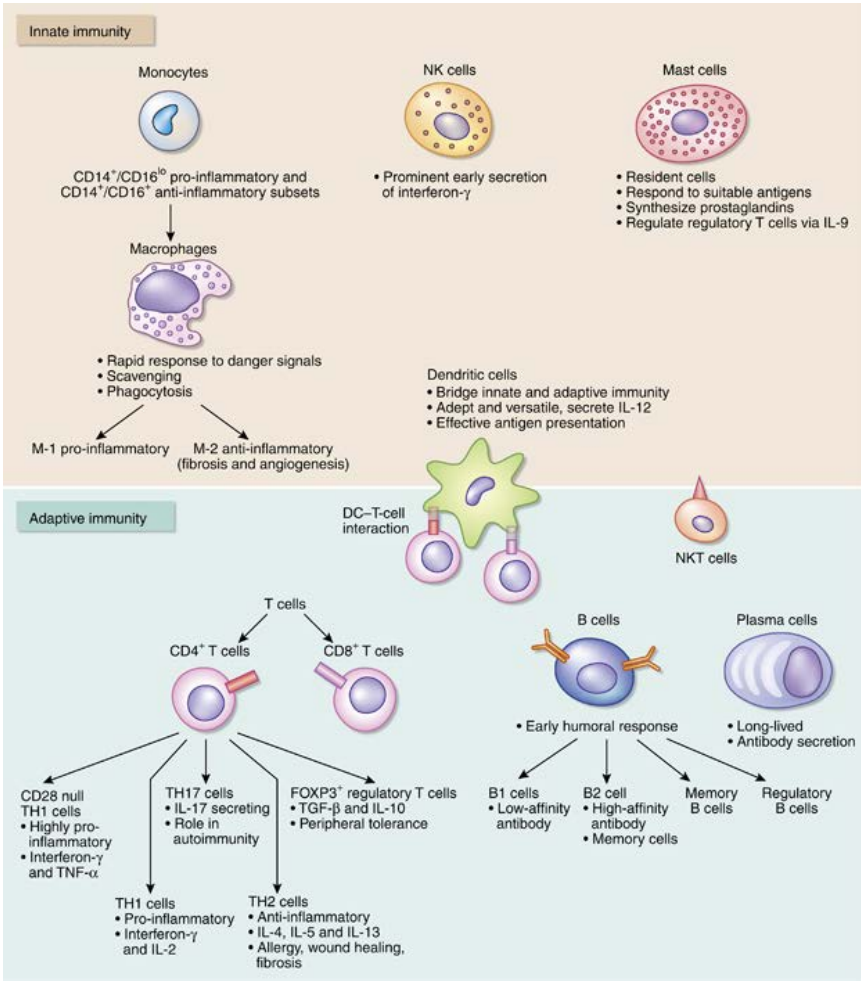
T cells have been detected in both human and mouse atherosclerotic plaques and in general, the majority of plaque T cells are $CD4^+$ cells[95,96]. Interestingly, 10% of all T cells in the plaque were found to recognize oxLDL in an MHC class II restricted manner[97]. A detrimental role for $CD4^+$ T cells was observed after transfer of $CD4^+$ cells in immunodeficient $apoE^{-/-}$/scid/scid mice, which develop markedly increased atherosclerotic lesions[98]. The Th1 cell subset is thought to exert pro-atherogenic effects via the excretion of its principal cytokine IFNγ. Mice deficient for IFNγ develop less atherosclerosis[99], however, it must be mentioned that other cells implicated in atherosclerosis, namely NKT cells and NK cells, also secrete IFNγ. Conversely, Th2 cells are described to have both pro- and anti-atherogenic properties. For instance, Th2 cells secrete IL-4, and deficiency of IL-4 was shown to result in decreased atherosclerosis[100]. However, Th2 cells are also an important source of IL-5, which promotes the differentiation of atheroprotective B1 cells[101]. The subset Th17 cells are postulated to aggravate atherosclerosis, mediated by the secretion of the proinflammatory cytokine IL-17. However, no definite conclusions can be drawn on the effects of IL-17, considering some groups showed that blocking IL-17 reduced lesion formation[102], while others found no effects on plaque burden[103]. Regulatory T cells are important for immune suppression and consequently, they are thought to be beneficial in the context of atherosclerosis. Indeed, depletion of $CD4^+Foxp3^+$ cells in $apoE^{-/-}$ mice results in increased atherosclerotic lesion formation. The effect of depleting $CD8^+$ T cells has been under debate, and although it has been previously reported that $CD8^+$ cells do not affect lesion size[104], recent data implicate proinflammatory $CD8^+$ cells in plaque destabilization[105].

B cells

B cells and the wide variety of antibodies directed against an extensive amount of diverse pathogens produced by these cells form a central part of the humoral immune response. In the bone marrow, B cells are formed from hematopoietic precursor cells after a series of functional rearrangements of immunoglobulin gene segments[106]. Eventually, an IgM molecule is expressed on the surface of these cells, which are then named "immature B cells". Immature B cells migrate to the spleen after they have left the bone marrow, where they differentiate into naïve, follicular or marginal zone B cells[107].

Relatively few B cells are found in the atherosclerotic plaque; however, they are predominantly present in lymphocyte infiltrates in the adventitia called tertiary lymphoid organs[108]. In one of the first studies that demonstrated a role for B cells in atherosclerosis, surgical removal of the spleen in apoE$^{-/-}$ mice was found to aggravate lesion formation. Subsequent adoptive transfer of splenic B cells did not only reverse this effect, but even resulted in a mild protection against atherosclerosis[109], indicative of a protective effect of B cells. However, the depletion of mature B cells with the use of a CD20-specific monoclonal antibody unexpectedly reduced atherosclerosis[110]. These seemingly opposing roles of B cells may be partially explained by the differential role of the functional B cell subsets B1 and B2 cells. B1 cells produce natural antibodies such as IgM in a T cell independent manner, while B2 cells require T cell interaction for activation and antibody production such as IgG and IgE[111]. The transfer of specific B2 cells, but not the transfer of B1 cells, to lymphocyte-deficient triple knockout (TKO) mice was found to aggravate atherosclerosis[112]. Also, additional evidence for a detrimental role of B2 cells is provided by disruption of B cell activating factor (BAFF) signalling, which is necessary for the survival of B2 cells. Mice deficient for BAFF receptor lack B2 cells but have minor changes in B1 cells, which leads to reduced atherosclerotic lesion formation[113].

On the other hand, protective effects of the subset B1a cells have been demonstrated, which is thought to be caused by IgM deposits in the lesions[114]. Natural IgM may limit plaque burden by preventing uptake of oxLDL, inhibiting accumulation of apoptotic cells, and neutralizing inflammatory gene expression in response to oxidized lipids[115]. An additional third B cell subset, called the B10 cell, acts as negative regulator of the inflammatory response by producing IL-10[116]. Although a definite role for these regulatory B10 cells is not yet described in atherosclerosis, they are most likely to attenuate atherosclerosis via an immunosuppressive manner.



General overview of innate and adaptive immune cells and some of their key effector functions. Adapted from Swaminathan *et al*. Kidney Int. 2011;80:453-63.

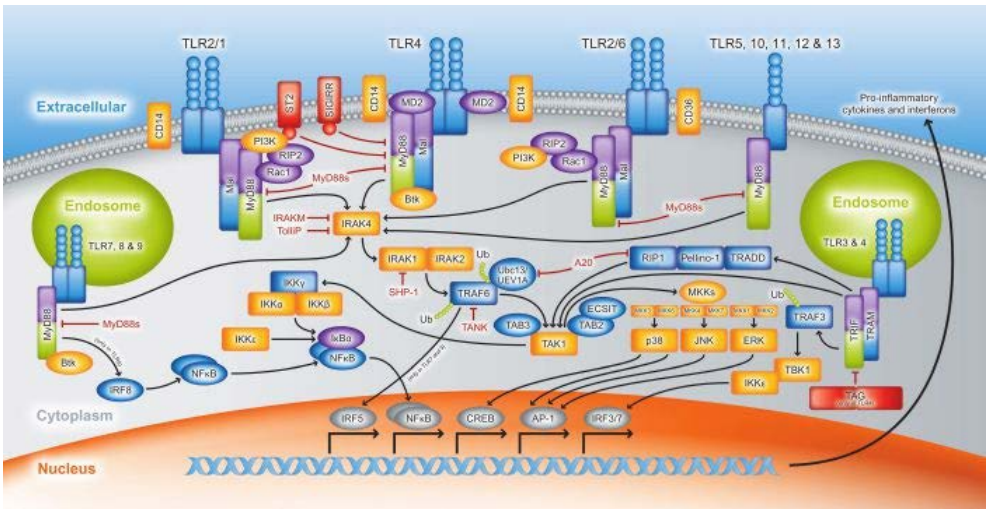
Toll-like receptor signalling

Toll-like receptors (TLRs) are a type of pattern recognition receptors (PRRs) that play a central role in the initiation of the innate and adaptive immune response. They are able to recognize conserved motifs on pathogens called pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) from endogenous damaged tissue[117]. In humans, 10 different TLRs have been identified (TLR1 to TLR10), whereas 12 distinct TLRs exist in mice (TLR1 to TLR9 and TLR11 to TLR13)[118]. TLR1 and TLR2, both expressed on the plasma membrane, can heterodimerize and subsequently sense bacterial triacylated lipopeptides. TLR2 is also able to heterodimerize with TLR6, which can recognize bacterial diacylated lipopeptides[119]. TLR3 is localized in the endolysosomal

compartment and recognizes double stranded RNA. TLR4 and TLR5, both present on the cellular membrane, can be activated respectively by lipopolysaccharide (LPS) and flagellin from bacteria[120]. TLR7, TLR8 and TLR9 are, similar to TLR3, located intracellularly. TLR7 and TLR8 sense single stranded RNA while TLR9 binds unmethylated bacterial CpG DNA[121].

Upon binding of a ligand to the TLR, adaptor proteins are recruited to the cytoplasmic domain of the receptor. All TLRs, with exception of TLR3, interact with MyD88 via their TIR domain, which results in a series of intracellular signalling events leading to translocation of NFκB to the nucleus[122]. TLR4 and the intracellular TLRs may also signal via an MyD88 independent pathway, which ultimately results in type I Interferon (IFN) production[121].

In one of the earliest studies investigating the role of TLRs in atherosclerosis, lesions were induced in MyD88 deficient mice. These mice developed reduced atherosclerotic plaques, caused by decreased macrophage recruitment to the lesion due to lower chemokine levels[123]. These findings were confirmed by Michelsen *et al.*[124], who also demonstrated that lack of TLR4 decreases atherosclerosis. TLR4 is the most extensively studied TLR regarding atherosclerotic research, and will be discussed into more detail in the next section. Similar to TLR4, inactivation of TLR2 results in less macrophage recruitment and lipid accumulation to the lesion, thereby attenuating plaque progression[125]. However, not all TLRs exert detrimental effects on lesion formation; for example, functional inactivation of TLR7 has been shown to result in accelerated atherosclerosis[126]. The exact role of other TLRs is still under debate, either due to lack of mechanistic data or due to conflicting evidence[127].



General overview of the extra- and intra-cellular Toll-like receptors and their signalling pathways. Adapted from Abcam Posters.

TLR4 and its accessory molecule RP105

Signalling via TLR4 requires the presence of the soluble protein MD2, which is associated with the extracellular domain of TLR4. In contrast to other TLRs, TLR4 does not directly bind to its ligand. Instead, MD2 is the primary recognition molecule responsible for the interaction with LPS[128]. The responsiveness to LPS is enhanced by the cell-surface protein CD14; CD14 can bind LPS and mediate its transfer to the TLR4/MD2 complex[129]. A distinct regulatory molecule, radioprotective 105 (RP105), was initially thought to act as an enhancer of TLR4 signalling as well. RP105 was discovered on the cell surface of B cells, in which it was found to drive B cell proliferation and activation[130,131]. Later, it was discovered that RP105 is not B cell specific, but that it is expressed by all myeloid cells. The structure of RP105 is highly comparable to that of TLR4 and similar to the TLR4/MD2 complex, RP105 associates with an adaptor molecule: MD1. However, the RP105/MD1 complex lacks the intracellular TIR domain, suggesting it may not induce signalling by itself[132]. In contrast to its stimulatory role on B cells, RP105 was found to inhibit TLR4 mediated responses in dendritic cells and macrophages[133]. Consequently, LPS injection in RP105 deficient mice results in an exaggerated inflammatory response with profoundly increased TNFα plasma levels[134].

Besides LPS, TLR4 has many other ligands that may induce inflammatory signalling. Interestingly, several of these endogenous ligands are present in the atherosclerotic plaque, such as oxidized LDL, heat shock protein 60 (Hsp60) and the alternatively spliced EDA (extra domain A) of fibronectin[135,136]. Targeting of TLR4 in atherosclerosis, as well as in vein graft disease, has thus shown some promising results, mostly via inhibition of vascular inflammation[124,137–139]. The effects of RP105 deficiency in cardiovascular disorders are less straight-forward. In a setting of damage-induced vascular remodelling, lack of RP105 results in increased neointima formation, which is in line with the inhibitory effects RP105 can exert on TLR4 in dendritic cells and macrophages[140]. However, reduced atherosclerotic lesion formation is observed in LDLr$^{-/-}$ mice on a high fat diet receiving RP105 deficient bone marrow, due to a reduction in inflammatory B2 cell numbers as well as decreased activation of B2 cells[141]. These conflicting data reflect the dichotomous effects RP105 can have on different cells types, and future research is aimed at further elucidating the regulatory mechanisms of RP105 in vascular remodelling.

The complement system

The complement system consists of over 30 plasma proteins that are converted into active proteases upon activation. Although complement is considered a part of the innate immune system, it may serve as a functional bridge between innate and adaptive immunity via enhancing antibody responses and immunological memory[142]. Complement activation can occur via three major pathways: the

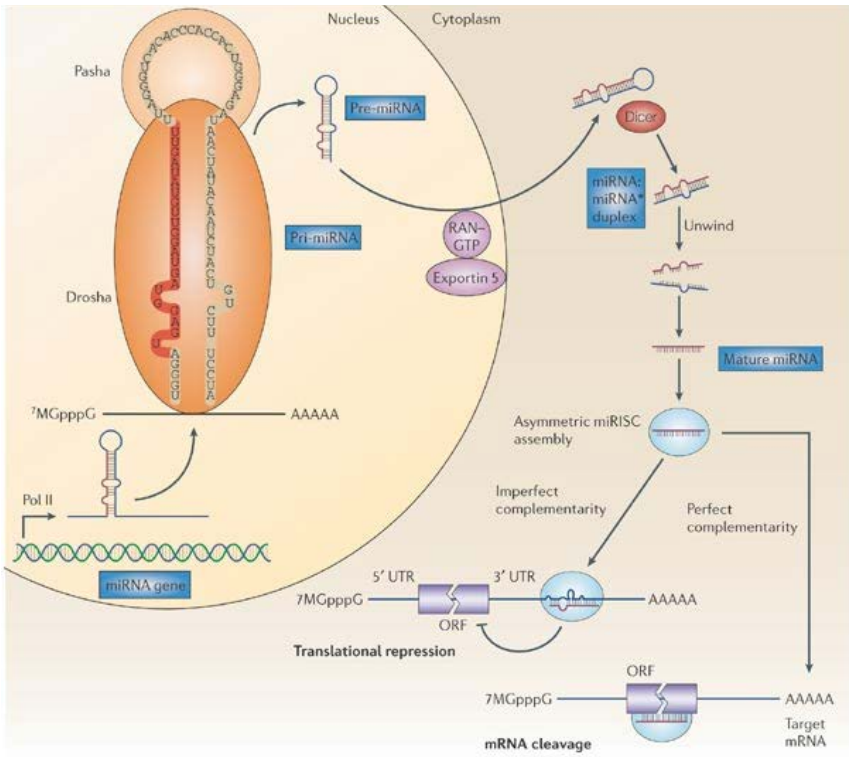
classical, the lectin or the alternative pathway; and also via the 'coagulation' pathway, which all result in the initiation of the complement cascade. The classical pathway is activated by binding of antibodies to their target antigens, while the lectin pathway is induced by binding of mannose-binding lectin to mannose residues on a range of bacteria[143]. The alternative pathway on the other hand is spontaneously activated at a constant low level. Finally, the 'coagulation' pathway is triggered by the cleavage and immediate activation of C3 and C5 by thrombin[144]. All major pathways lead to the formation of convertases that cleave the central component C3 into C3b and C3a. C3b is responsible for the amplification reaction of the complement cascade and the anaphylatoxin C3a is a potent chemoattractant that induces proinflammatory signalling after binding to its receptor C3aR. Cleavage of C5 results in the formation of C5b, which initiates the assembly of the terminal complement complex (TCC). Also, C5a is formed, a powerful anaphylatoxin with chemoattractant and inflammatory properties similar to C3a[145,146]. To prevent excessive activation and damage to healthy tissues, multiple proteins regulate the complement cascade, including factor H, CD59 and DAF[143].

Complement activation in the context of atherosclerosis may be induced by different mechanisms, for instance via immune complexes, c-reactive protein (CRP), apoptotic cells and modified LDL[147]. Also, the expression of receptors for C5a and C3a is significantly upregulated in human coronary plaques compared to normal coronary arteries[148]. However, the exact contribution of the complement system to atherosclerosis is not yet elucidated. Deficiency of the central component C3 aggravates aortic lesion development in mice placed on a high fat diet[149,150], while the opposite effect was found when targeting of C3 in the setting of vein graft disease[151]. Other studies point to a protective role for the inhibitory proteins CD59[152] and DAF[153], while the complement component C6[154] exerts detrimental effects on atherosclerosis. Of particular interest is the anaphylatoxin C5a, which has been shown to associate with future cardiovascular events in patients with advanced atherosclerosis[155]. C5a may aggravate plaque formation and destabilization via activation of endothelium, attraction of leukocytes to the site of inflammation and inducing the expression of multiple inflammatory cytokines. Further investigations into the exact role of C5a in atherosclerosis is therefore of high interest.

MicroRNAs

The presence of microRNAs (miRs) in vertebrates was first described by three separate groups in a series of Science papers in 2001[156–158]. Each of these papers defined miRs as short, noncoding RNA strands approximately 21-25 nucleotides in length, capable of posttranscriptional regulation of specific mRNA targets. The posttranscriptional repression is induced by Watson-Crick base pairing between

the miR seed region and sequences located in the 3' untranslated region (UTRs) of the mRNA targets. Following these first pioneering papers, a burst of studies into the biological actions of miRs in different research areas has ensued.



MicroRNAs are generally transcribed in the nucleus as large pri-miRNA transcripts. Processing by the enzyme Drosha results in the formation of pre-miRNA, which is transported into the cytoplasm and further processed by the Dicer enzyme. The miRNA is loaded into the RNA-induced silencing complex (RISC), where it can bind to the complementary site of the mRNA target, thereby leading to either translational repression or mRNA cleavage, depending on the degree of complementarity. Adapted from Esquela-Kerscher *et al.* Nat rev cancer. 2006;6:259-69.

The distinct characteristic of miRs to regulate the expression of numerous genes makes them interesting targets for interference in complex, multifactorial disease-processes. Indeed, several studies have shown that inhibition of specific miRs in atherosclerosis leads to profound effects on lesion development or progression. One of the most extensively investigated miRs with regard to atherosclerosis is miR-33: a miR encoded by the intron of sterol regulatory element-binding protein 2 (Srebp2), which is important for cholesterol synthesis and uptake. Mice deficient for miR-33 were shown to have increased ABCA1 levels in the liver and higher serum HDL levels[159]. These findings were confirmed in primates, in which inhibition of miR-33a/b resulted in increased HDL levels and also reduced VLDL levels[160]. However, the exact role of miR-33 in atherosclerosis has recently been under

debate. While several papers showed that antagonism of miR-33 induces plaque regression or prevent lesion progression[159,161,162], others have recently claimed that prolonged silencing of miR-33 fails to induce a sustained increase of HDL levels and does not prevent plaque progression[163]. A different miR implicated in atherosclerosis, miR-155, is involved in inflammatory signalling in macrophages, endothelial cells and smooth muscle cells. Deficiency of miR-155 was found to reduce the expression of CCL2 while increasing Bcl6 expression, a transcription factor that represses proinflammatory NFκB signalling, leading to aggravated atherosclerotic lesion formation[164]. Conflicting *in vitro* evidence however, shows that miR-155 deficient macrophages stimulated by oxidized LDL display an enhanced inflammatory response[165]. Besides miR-33 and miR-155, several other miRs have been described to be either present in lesions or to be able to affect lesion formation.

The above mentioned examples of miR inhibition in the setting of atherosclerosis clearly demonstrate the potential therapeutic value miRs may have. However, it has also become evident that miR research is still a developing field with accompanying shortcomings and contradictory reports. Moreover, the unique ability of miRs to regulate a biological process via a multitude of genes rather than single genes has not been utilized to its full extent yet. Therefore, future research directed towards validating known miRs, as well as identifying miRs not yet described in atherosclerosis, may yield promising results.

Outline of this thesis

The aim of this thesis is to gain more insight into the role of the innate immune system in vascular remodelling, in particular in vein graft disease and atherosclerosis, as well as to identify new therapeutic targets to prevent vascular diseases and acute cardiovascular syndromes. **Chapter 2** encompasses a review which focuses on the role of the mast cell in early and late stage atherosclerosis, with particular emphasis on plaque rupture. Also, the prognostic value of mast cell mediators as well as therapeutic implications of mast cell stabilization are discussed. While mast cells have previously been indicated as important players in atherosclerosis, their role in vein graft disease has not been elucidated yet. In **chapter 3**, we show that mast cell activation results in a marked increase in vein graft thickening and plaque disruptions. Also, we investigated the mechanism via which mast cells are activated in the context of vein graft disease. We found that the complement component C5a, capable of activating mast cells, is upregulated during vein graft disease. Local application of C5a in a gel aggravated vein graft disease, which could be inhibited with the mast cell stabilizer cromolyn, indeed indicating a mast cell dependent mechanism. **Chapter 4** describes the effect of

C5a on advanced vein graft lesions. Application of C5a in these late stage lesions increased plaque disruptions in a mast cell independent manner, since cromolyn was not able to reduce these events. Instead, C5a was seen to directly promote apoptosis of smooth muscle cells and endothelial cells *in vitro* and *in vivo,* which may overrule the effect of the mast cells and lead to the observed increase in disruptions. **Chapter 5** focuses on the role of mast cell derived chemokines in atherosclerosis. Chronic mast cell activation results in a profound increase of neutrophils in the intima and adventitia of atherosclerotic plaques. *In vivo* influx studies using mast cell deficient mice and control mice show indeed that mast cell activation leads to excessive neutrophil migration.

Chapter 6 provides insight in the role of RP105 in vein graft disease. Deficiency of RP105 in mice fed a chow diet exacerbates vessel wall thickening of the vein grafts, as well as the number of plaque disruptions. An additional *in vivo* study shows that $LDLr^{-/-}/RP105^{-/-}$ mice on a high fat diet do not display increased vessel wall thickening compared to control $LDLr^{-/-}$ mice, however more plaque disruptions were still observed. Both studies show that RP105 deficiency increases the number of activated perivascular mast cells, which was more pronounced in mice fed a chow diet, thereby possible leading to the observed effects of vein graft disease. In **chapter 7,** total body RP105 deficiency in atherosclerosis has been investigated. Opposed to vein graft disease, $LDLr^{-/-}/RP105^{-/-}$ mice on a high fat diet develop reduced early atherosclerotic lesions, accompanied by a decrease in lesional macrophages. We demonstrate that this may be caused by disturbed monocyte migration in RP105 deficient mice, due to a downregulation of the chemokine receptor CCR2.

Chapter 8 describes the use of a Reversed Target Prediction (RTP) method to identify microRNAs that are predicted to affect atherosclerotic lesion development and thus act as a so-called master switch. With this RTP, we singled out miR-494 from the miR-gene cluster 14q32, which has not been previously described in atherosclerosis. Inhibition of miR-494 *in vivo* by means of gene silencing oligonucleotides (GSOs) results in reduced lesion formation, while plaque stability is increased. Also, upregulation of target genes for miR-494 is observed both *in vivo* and *in vitro*.

The results of all the studies described in this thesis, as well as future prospectives, are discussed in **chapter 9.**

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