Mast cells as immune regulators in atherosclerosis

Kritikou, E.

Mast cells as immune regulators in atherosclerosis

Eva Kritikou

MAST CELLS AS IMMUNE REGULATORS IN ATHEROSCLEROSIS

Eva Kritikou

12 December 2017

Institute: Leiden Academic Centre for Drug Research (LACDR)

ISBN: 978-90-9030633-9

Printer: PRINTRS B.V.

Proefschrift Leiden
Met literatuur opgave – met samenvatting in het Nederlands

Mast cells as immune regulators in atherosclerosis

PROEFSCHRIFT

Ter verkrijging van
de grad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus Prof. Mr. C.J.J.M. Stolker,
volgens besluit van het College voor Promoties
te verdedigen op dinsdag 12 december 2017
klokke 15.00 uur

door

Evangelia Kritikou

Geboren te Athene, Griekenland
in 1984

Promotor: Prof. dr. J. Kuiper
Co-promotor: Dr. I. Bot

Promotiecommissie:

	Prof. dr. H. Irth	- LACDR (voorzitter)
	Prof. dr. W. Jiskoot	- LACDR (secretaris)
Overige leden:	Prof. dr. C.J.M. de Vries	- AMC
	Prof. dr. P.H.A. Quax	- LUMC

The research described in this thesis was supported by a grant of the Dutch Heart Foundation (2012T083) and was performed at the Division of Biopharmaceutics, Leiden Academic Centre for Drug Research, Leiden University, Leiden, The Netherlands. Financial support by the Dutch Heart Foundation for the publication of this thesis is gratefully acknowledged.

The realization of this thesis was also financially supported by Leiden University.

"Άλλά κάτεχε ότι μονάχα κείνος
που παλεύει το σκοτάδι μέσα του
θα 'χει μεθαύριο μερτικό δικό του στον ήλιο."

Το Άξιον Εστί
Οδυσσέας Ελύτης
Νόμπελ Λογοτεχνίας , 1979

"But you should know that only he
who wrestles with his inner darkness
will tomorrow have his place in the sun."

Axion Esti (Worthy it is)
Odysseus Elytis
Nobel Laureate in Literature, 1979

Table of Contents

Chapter 1

General introduction - Atherosclerosis

A brief history of atherosclerosis

In the course of human history, infectious diseases, spread by pathogenic micro-organisms, are held accountable for the loss of millions worldwide[1]. The rise of the 20th century however, found humankind in the brink of medical breakthrough with vaccine development[2] dramatically reducing mortality rates, while life expectancy was increased from a two decades-span to 80 years[1,3]. Yet, along with medical and technological prosperity came the Western-world model of life, accompanied by chronic pathological conditions like cancer, Alzheimer's and cardiovascular disease[4].

Cardiovascular disease is the principal cause of death in modern society, with 44% of all adults in the United States estimated to develop some form of cardiovascular disorder by year 2030[5]. Among the different cardiovascular syndromes, the highest mortality rate is observed in coronary heart disease, with myocardial infarction being its most well-known manifestation[5,6]. Acute coronary syndromes have a common main underlying pathology; atherosclerosis[7]. Atherosclerotic disease has been described in humans since the ancient years[8], but it is the modern-era nutritional habits[9] that deem it the prevailing component of human mortality[10].

It was in 1907 when the Russian scientist Alexander I. Ignatowski reported that rabbits being fed a high-fat diet, develop atherosclerotic disease in their aorta[11]. A few years later, in 1913, Nikolai N. Anitschkow revealed that the component responsible for the development of atherosclerosis was the high cholesterol content of the high-fat diet[12]. Yet, it took until the 1980s to fully assess the contribution of the immune system in the form of inflammatory monocytes on the disease progression[13,14,15]. From that point and to this day, the research community has advanced greatly in understanding the pathology of atherosclerosis[16,17]. Up to now however, the field is still divided over whether atherosclerosis is a lipid or an immune-mediated disease, giving rise to the ongoing debate of "*the lipid versus the immune hypothesis*".

1. Atherosclerosis pathology

Atherosclerosis is a multifactorial disease with a complex background and a chronic, yet silent mode of action; it begins through an individual's early teenage years and can remain undetected for decades until clinical symptoms, requiring an expert's intervention, appear[18]. The pathology of atherosclerosis is established with the formation of multiple lipid-rich plaques in the large branches of the arterial tree[19]. The most susceptible sites within the human body are the ones most sensitive to shear stress changes[20] such as the carotid bifurcation, aortic arch, abdominal aorta and coronary arteries of the heart.

The arterial wall is paved with a layer of tightly packed endothelial cells (ECs), to assist normal vascular tone, nutrient permeability to the tissues and blood distribution over the different body compartments[21]. Disturbed blood flow, in the presence of increased serum (very) low density lipoprotein (V/LDL)[22,23], can induce damage on the endothelial cell bed[24,25] resulting among others, in modulation of the EC tight junctions through molecules like PE-CAM-1 and VE-cadherin[26] and upregulation of adhesion molecules such as VCAM-1 or ICAM-1[27]. The damage inflicted upon the endothelial cell junctions permits the lipoprotein molecules to infiltrate the subendothelial space[28], bind proteoglycan molecules[29,30] and undergo chemical modifications[31]. This process retains the lipoprotein molecules in the subendothelial space. Concurrently, loss of the vascular EC pattern[32] and adhesion molecule upregulation[33] but also chemokine secretion upon EC activation[34], initiates the permeation of inflammatory immune cells in the area[17]. Accumulated phagocytes are able to digest the modified LDL molecules giving rise to a thin intimal layer called fatty streak[35]. Fatty streaks can be detected already at an infant age, usually depending on the genetic and environmental background of the mother[36]. These early atheromata are harmless and can disappear during development, for instance by lowering serum cholesterol levels[37]. In fact, through an individual's life initial-phase atherosclerotic plaques can appear and disappear multiple times. However, increasing lipid deposition over time at a pre-established atherosclerotic site leads to higher plaque volume and advanced atherosclerotic disease stages[38].

The point of advancement from a thin-intimal layer to a progressed atherosclerotic plaque depends greatly on the function of phagocytes. Upon increased non-resolved inflammation in the plaque area, phagocytic cells become apoptotic[39,40]. In an attempt to clear the apoptotic material accumulating within the vessel wall, a process termed efferocytosis takes place[41]. However, as more and more lipids accumulate in the area, this programmed apoptotic mechanism begins to fail, while being replaced by non-specific secondary necrosis[42]. Necrotic material accumulation and destruction of the cellular plaque composition leaves behind debris, termed necrotic core regions[43,44], and increased plaque burden[45]. Upon further progression, the atherosclerotic plaque is also characterized by an increase in smooth muscle cell content[46], extracellular matrix protein synthesis and degradation[47] and neovessel formation[48]. Smooth muscle cells (SMCs) are mainly considered protective, by producing extracellular material in the form of collagen-rich fibers that stabilize the plaques[49,50]. In addition, SMCs can increase their proliferation rate and migrate on the outskirt of the plaque, to form a protective fibrous cap[51,52]. On the contrary, intraplaque neovascularization, which arises from the hypoxic conditions present in the plaque[53], increases the inflammatory burden in the area, by giving passage to immune cell infiltration inside the intima[54]. Furthermore, the plaque site is subjected to a high risk for microvessel leakage which can lead to intraplaque hemorrhage[55]. Advanced plaques do not have the ability to recede[37], increasing thus

the chances for an acute cardiovascular event, particularly when they reach the critical stage of a fibroatheroma[56,57]. Uncontrollable progression of a plaque can cause an artery to become stenotic and disturb normal blood flow. An advanced plaque can get eroded or rupture, causing the formation of a thrombus[58,59]. Formation of a thrombus at the ruptured site can cause vessel occlusion and result in a myocardial infarction episode[60] (**Figure 1**). However, a thrombus can also get destabilized, and travel to distant narrower vessels, obstructing thus the blood supply and resulting in impaired tissue oxygenation in the form of a stroke[61]. At this point, the disease has rapidly progressed to an acute clinical stage whereupon surgical intervention is demanded.

General intervention strategies include endarterectomy surgery to remove the plaque[62], as well as balloon angioplasty, with or without stent placement[63,64], to restore the normal blood flow; alternatively, vascular bypass operation[65] helps to divert the blood circulation *via* a non-occluded vessel. Yet, all these intervention methods usually lead to complications, like restenosis[66]. In the case of atherosclerosis diagnosed prior to clinical manifestation of a cardiac syndrome, pharmacological treatment through statin use can decrease the (V)LDL content, providing plaque stability[67,68]. However, lipoprotein reduction alone, without taking the inflammatory context into account, is not able to lower the plaque burden and lead to atherosclerotic plaque regression[69].

Interestingly, not all late stage plaques cause clinical events. In fact the plaque characteristics that lead to an acute cardiac event appear to shift nowadays from the classical stratification[70] and at the moment it is unknown why some plaques appear "vulnerable" enough as to rupture, yet may not cause clinical symptoms, while plaque erosion can lead to acute events without rupture[71].

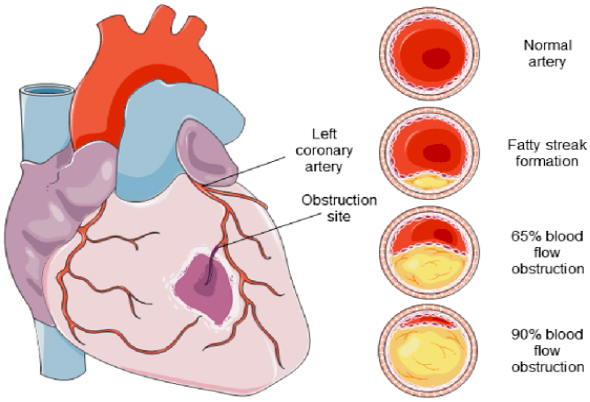


Figure 1: Myocardial infarction site. Coronary arteries show high preponderance in atherosclerotic plaque formation. Fatty streaks are harmless intima protrusions with reversible plaque formation. However, increasing lipid deposition leads to advanced plaques, or fibroatheromas, characterized by necrotic cores and surrounded by a fibrous cap. Advanced plaques tend to either obstruct the blood flow or rupture and release their highly thrombotic material to the lumen, increasing the chance for an ischemic episode at a narrower vessel.

Finally, it is important to remember that the progression rate of atherosclerosis, aside from the obvious link between high cholesterol levels and poor dietary choices[9], also depends on various determinants acting independently or simultaneously, such as diabetes[72], hypertension[73], smoking[74] or even an individual's gender[75]. All the above indicate the complex nature of the disease and how crucial it is to control atherosclerosis development at an early stage.

Our current knowledge on the processes that shape atherosclerosis, derives mainly from experimental animal models or from observational studies of human material collected at the end-stage of the disease. Of the animals used, the most common are genetically modified mouse models[76]. Rabbits[77] or non-human primates[78] are models that translate more accurately to the human condition, since rodents do not develop spontaneous atherosclerosis; yet, the accelerated pace of atherosclerosis development in mice, as well as the fast breeding and relative ease in genetic modulation, grants mice useful to study of lipid-induced atherosclerosis. However, the translation of mouse atherosclerotic disease to the human situation is as if referring to the hereditary condition of familial hypercholesterolemia[79]; an accelerated atherosclerosis syndrome which occurs only in a small proportion of the population with plaques showing differential characteristics as compared to the end-stage atherosclerotic tissue collected in most human studies[80]. A typical example of the difference between mouse and human atherosclerosis is that mouse plaques rarely advance to an end-stage and cannot form spontaneous thrombi[81]; two processes that are not only typical in human atherosclerosis but also, as explained above, the most crucial phase of the disease. It is therefore important to remember that extrapolation of mouse research into the human condition is not always a straight line. Yet, mouse atherosclerotic plaques still show many common characteristics with human plaques and are useful to study the mechanisms underlying specific pathways, as well as to develop new therapeutic interventions.

2. Lipid-induced responses

Circulation of cholesterol particles at normal levels is a physiological process required for steroid hormone production[82], bile acid synthesis[83] and cell membrane structure[84]. Plasma cholesterol levels are influenced, up to an extent, by the cholesterol amount absorbed through the diet[85]. Dietary cholesterol absorption is mediated in part by the intestines, the function of which has gained increasing attention in the field of atherosclerosis[86]. However, cholesterol homeostasis is mainly regulated by the liver[87], a key organ responsible for its synthesis and degradation[88]. In brief, the synthesis of cholesterol is controlled by SREBP-proteins[89] and the enzyme HMG-CoA reductase[90]. Cholesterol molecules are delivered to the blood in the form of VLDL or its hydrolyzed product, LDL[91] (**Figure 2**). Excess low-density lipoprotein particles are controlled by

the hepatic LDL-receptors (LDLr)[92], which are responsible for their uptake, thereby reducing blood cholesterol levels. The endocytosed cholesterol is broken down into bile acids and transferred to the intestines for excretion[93].

When cholesterol levels in the cell increase, the transcription factor LXR launches a complex regulation pathway which aims to reduce cholesterol production and uptake, but also increase the excretion of cholesterol from the cell[94]. For example, LXR can inhibit the uptake of cholesterol by negatively regulating the expression of LDLr[95], can interrupt cholesterol synthesis by acting on the SREBPs[96], but can also increase the transcription of the cholesterol efflux proteins ABCA1[97] and ABCG1[98] which release the accumulated intracellular cholesterol. The action of LXR on ABCA1[99] and ABCG1[100] results in the production of high-density lipoprotein (HDL) particles. Hepatic cholesterol is released through ABCA1 in the form of apolipoprotein-AI and apolipoprotein-E particles, that later assemble into HDL[101].

The function of HDL is considered mainly beneficial[102], with high serum levels long being proven as protective against the development of cardiovascular syndromes[103,104]. In addition, increased HDL and apoAI have been proven indispensable during atherosclerotic plaque regression[105–107]. This grants HDL the name "good" cholesterol. On the contrary, excessive levels of "bad" cholesterol or (V)LDL in the blood have, as mentioned above, been associated with the prevalence of coronary heart disease[108,109], while non-HDL cholesterol reduction has been proven time and again to lower the clinical risk for atherosclerosis-mediated cardiac syndromes[110,111]. In fact, elevated serum LDL levels are associated with atherosclerotic plaque progression[112].

Specifically, lipoprotein molecules that have already penetrated the endothelial cell barrier, and have been further subjected to modifications such as oxidation[31], are subsequently taken up by locally proliferating macrophages, which then become foam cells[113]. Intraplaque macrophages internalize oxidized LDL through receptors such as protein CD36, that serves as a scavenger receptor[114]. The uptake of LDL by foam cells leads to a circle with an end-goal to process the cholesterol molecules so as to be less toxic for the tissue and therefore reverse the local damage. This begins by the intracellular break-down of cholesterol into cholesteryl esters, in order for the cells to battle its toxicity[115]. Cholesterol esterification is followed by circles of hydrolysis which prepare the molecules for efflux from the cell[116]. Free cholesterol is released from the cells through ABCA1 and ABCG1 and is removed from the arterial wall and back into the liver as HDL-apoAI particles[117], in a process named reverse cholesterol transport[118,119]. These particles can then be internalized by the scavenger receptor SR-BI in the liver[120] and subsequently get cleared away by the intestines[121]. However, as lipid accumulation in the cell persists, reverse cholesterol transport fails and the internalized free cholesterol becomes toxic for the cell, leading thus to foam cell apoptosis[122]. As explained before,

incessant local apoptosis intensifies the already initiated inflammatory response in the arterial wall leading thus to the formation of advanced atherosclerotic plaques[41].

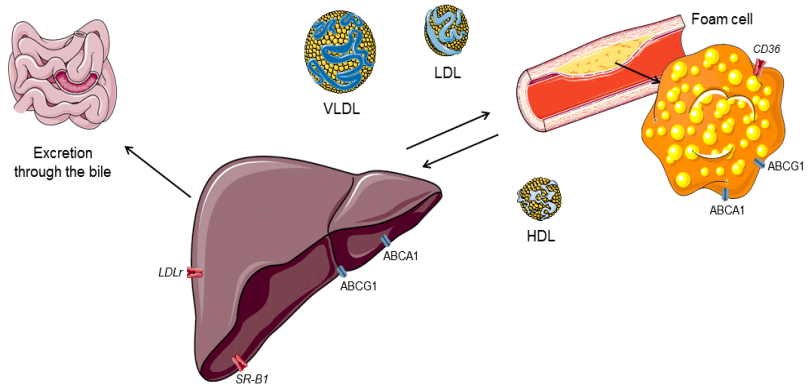


Figure 2: Cholesterol metabolism pathway. Cholesterol is synthesized in the liver and released in the circulation *via* (V)LDL molecules. High concentration of serum cholesterol leads to the accumulation of low-density lipoprotein particles in the subendothelial space where they get modified and internalized by foam cells, through protein *CD36*. Cholesteryl esters are intracellularly broken down into free cholesterol which is then released *via* the efflux receptors ABCA1/G1. The free cholesterol molecules are packed into HDL particles that can be transferred to the liver. Lipoproteins are being internalized in the liver through receptors *LDLr* and *SR-BI* and cholesterol is further degraded into bile acids which are transported into the intestines for excretion. *Abbreviations: (V)LDL: (very) low-density lipoprotein, HDL: high-density lipoprotein, LDLr: low-density lipoprotein receptor, CD36: cluster of differentiation 36, ABCA1/G1: ATP-binding cassete A1/G1, SR-BI: scavenger receptor B type I.*

The discovery of the cholesterol regulation pathway has developed into an important tool in atherosclerosis research, since the most common experimental mouse models used to study the disease are the LDL-receptor-deficient ($LDLr^{-/-}$) and apoE-deficient ($apoE^{-/-}$) mice[76]. The function of both models relies on the increased circulation of V/LDL particles, which generates atherosclerosis in the arterial tree, primarily the aortic arch and root. Specifically, the $apoE^{-/-}$ mice lack apolipoprotein E particles and therefore VLDL cannot be cleared away by the liver, with these mice showing spontaneous hyperlipidemia and atherosclerotic plaque formation[123]. In contrast, the $LDLr^{-/-}$ model does not develop atherosclerosis unless placed on a high-fat diet, whereupon V/LDL is retained at high concentrations in the blood since it cannot bind the LDL-receptor and get cleared away through the liver[124]. Both models show many similarities in the development of atherosclerosis. However, it is important to note that they can also have crucial differences, as for example in certain immune system-mediated pathways[125]. It is important to keep this in mind[126], particularly when it comes to the regulation of subtle responses that may however fine-tune crucial pathways.

Overall, for cholesterol to be optimally regulated through the body, the liver, the intestines and the circulatory system are at a close interrelated communication;

malfunction of a process in one of these compartments can influence the other and thus disturb cholesterol homeostasis and increase the risk for cardiovascular disease[127].

3. The role of the immune system

It is apparent that serum cholesterol deregulation is a major component for the initiation of atherosclerosis. However, it is the dysfunction of the immune system that propagates the disease into an advanced stage[128]. The immune system response generally, as well as in atherosclerosis, is classified into a fast reaction elicited by innate immune cells[129], such as neutrophils, monocytes and mast cells, and a slower, more specific, reaction through adaptive immune T and B lymphocytes and NKT cells[130] (**Figure 3**). Most immune cell types play a detrimental role in the plaque development, however the remarkable aspect of the immune system is that while most cell actions augment atherosclerosis, there are certain immune cell subsets and pathways that upon manipulation appear to act in a protective manner against it[131].

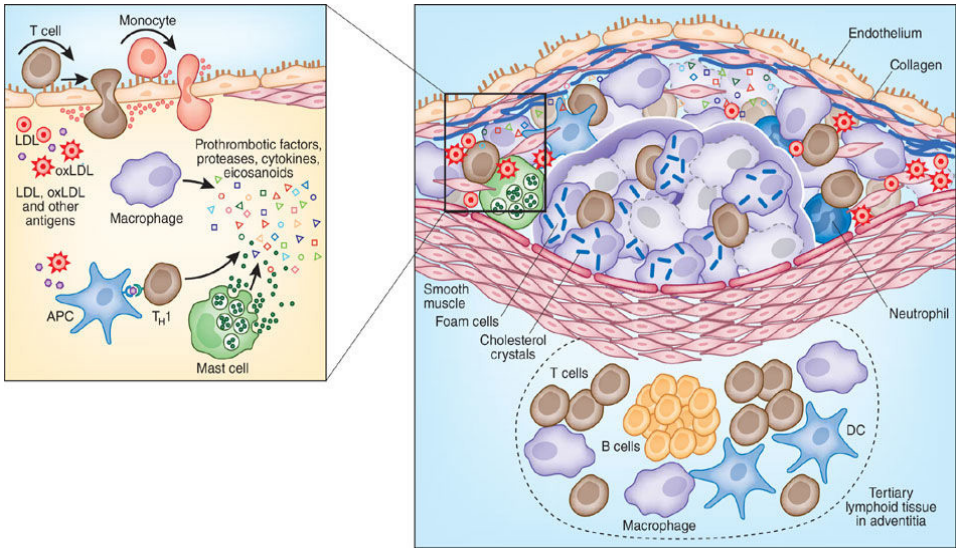


Figure 3: Immune cell types involved in atherosclerosis. The main cellular constituents of an atherosclerotic plaque are the macrophages. Macrophages proliferate locally in the plaque after their monocytic precursors penetrate the endothelial wall layer. Macrophages are able to internalize oxidized LDL, thus becoming foam cells. Other innate immune cells, like neutrophils and mast cells, also infiltrate the subendothelial space and release their granular contents in the area. Secreted pro-inflammatory cytokines and proteases enhance the endothelial damage and break down collagen fibers, intensifying thus the local inflammation. Macrophages and dendritic cells (DCs) can also drive the adaptive immune response, by processing and presenting lipid antigens to T cells and B cells in secondary lymphoid organs. Lymphocytes initiate lipid specific responses that mainly propagate the inflammatory cascade, resulting thus in apoptosis, necrosis and destabilization of the atherosclerotic plaque. *Adapted from Hansson & Hermansson, Nat. Immunol. 2011; 12, 204-212*

3.1 Macrophages

As discussed, macrophages, through their capacity to ingest lipoprotein particles, are the major immune cell type within atherosclerotic plaques, both in quantity and importance. Macrophages appear within the plaque upon differentiation from locally infiltrated monocytes[132,133].

Monocytes are innate cells, generated from the bone marrow, which patrol the bloodstream in a guarding fashion and are ready to act upon sensing tissue damage[134]. Once a danger signal is received, monocytes will concentrate in the affected location, penetrate the endothelium and infiltrate the area in an attempt to control the damage[135]. In the case of atherosclerosis this happens as soon as lipoproteins accumulate in the subendothelial space[136]. Thereupon the damaged endothelial cells send out signals, for instance in the form of chemokine monocyte chemoattractant protein-1 (MCP-1/CCL2) among a complex array of chemokine signals[137]. MCP-1, as the name suggests, attracts monocytes in the plaque area, guided there by their expression of chemokine receptor CCR2[138]. Of note, the interaction between CCR2 and CCL2 is one of the most well described pathways in the generation of atherosclerosis[139.140]. Circulating monocytes can be divided into various subcategories, depending on their action and according to the expression pattern of certain proteins on their membrane[134]. In humans, monocytes are sorted into three classes based on the expression levels of proteins CD16 and CD14[141] with one of the subsets considered a predictor for future coronary events[142]. In mice, this classification relies on the expression of protein Ly6C[143]. In atherosclerosis, it has been proven that Nur77 is a key regulator of the anti-inflammatory Ly6C^{low} monocyte subset, since its deletion enhances pro-inflammatory monocyte responses and leads to atherosclerosis progression[144,145]. In contrast, Ly6C^{high} inflammatory monocytes circulate at an increased rate over high-fat diet, and drive atherosclerosis development[146] upon migration in the inflamed tissue. For example, Ly6C^{high} monocyte migration regulated by protein RP105[147] was shown to positively affect atherogenesis[148].

Once infiltrated within the subendothelial tissue, monocytes differentiate into plaque macrophages[149,150]. Uptake of (V)LDL molecules by macrophages results in the formation of lipid-laden foam cells[113,151]. The term “foam cell” derives from the distinct microscopic pattern of light-yellow lipid spheres that grant the cells a foamy appearance[152]. Foam cell formation, aside from its significance in cholesterol metabolism, affects also the immune response. Macrophages are cells designed to phagocytize foreign or toxic material within a tissue, to reduce the spread of the damage[153]. This is a process which activates the cells through specific receptors such as pattern recognition receptors, like the toll-like receptors (TLRs)[154]. Particularly, TLR4 has been found to bind oxLDL molecules, activate foam cells[155] and initiate a downstream cascade that

leads to the secretion of cytokines like IL-1β and IL-6, which further augment the inflammation in the tissue by acting on the T cells[156]. In addition, macrophages are a rich source of matrix metalloproteinases[156], which degrade the extracellular matrix of the plaque and affect plaque stability[157]. The phagocytic capacity of atherogenic proteins by macrophages, is proven to induce endoplasmic reticulum stress and thereby leads to cell apoptosis[158]. As mentioned before, induction of apoptosis during initial atherosclerosis stages can be beneficial, while persistent apoptosis at a later stage increases plaque development[159] and instability[160].

Macrophages appear in multiple subsets during atherosclerosis development, defined by their differential responses[161]. In the past, it was thought that macrophages are classified as either classical, inflammatory, M1 macrophages or alternative, healing, M2 macrophages[162]. However, more and more studies indicate that there are in fact multiple macrophage subsets and each one acts differently within the atherosclerotic plaque[163]. M1 macrophages were abundantly found in the plaques of patients suffering an ischemic episode, as compared to M2 macrophages which were increased in stable plaques[164]. In general, M1 macrophages are reported to enhance inflammation within the plaque, for instance through the production of toxic reactive oxygen species[165]. On the other hand, M2 macrophages have recently been proven responsible for atherosclerotic plaque regression[166]. This is the reason why therapeutic means for plaque reduction mainly aim on the action of macrophages. A third macrophage subset that seems to populate atherosclerotic plaques at high numbers are the Mox macrophages which are induced by accumulating oxidized lipids in the atherosclerotic environment and seem to be proatherogenic[167]. These three macrophage types appear to be the most frequent inside the atherosclerotic plaque. However, the macrophage classification scheme is a matter of debate. While some believe that macrophages are distinguished in many more subsets, according to their induction mechanisms and downstream properties[168], this is not a notion accepted by the entire scientific community. This further illustrates how plastic these cells can be, while highlighting the need for additional research in order to fully decipher the role of macrophages.

3.2 Dendritic cells

To make matters even more complex, monocytes can also differentiate into an additional innate cell subset, the dendritic cells (DCs)[169]. The difference between macrophages and DCs is an intricate matter, mainly due to their common share of most protein markers. The main difference lies in the expression levels of proteins CD11c[170] and F4/80[171] which are considered DC or macrophage-specific respectively. However, it rather seems that these two cell types share an interrelated plastic state because it has been shown that under inflammatory conditions, such as inside an atherosclerotic

plaque, macrophages upregulate the expression of CD11c[172,173], while DCs possess a cholesterol regulation machinery, take up lipoproteins and can become foam cells[174,175]. Interestingly, within the atherosclerotic plaque it is not just these two cell types that can become foam cells. Recently it was proven that smooth muscle cells also acquire a foam cell phenotype[176]. This indicates that local inflammatory pressure can exert atypical effects on the cell types involved. Particularly in the case of immune cells, their state and abilities are plastic enough as to alternate from one phenotype to the other.

Dendritic cells can also arise from hematopoietic progenitor cells and, similar to macrophages, develop into different subsets in atherosclerosis pathology[177]; the main being conventional (cDC) and plasmacytoid DCs (pDCs). cDCs can act in an anti-inflammatory manner, for instance through the control of Flt3, which was shown to be atheroprotective[178]. On the contrary, pDCs seem to affect atherosclerosis progression in a positive manner[179]. Importantly, while uptake of oxLDL by DCs is known to elicit a proinflammatory DC-action[180], the manner by which they take up and process LDL is very crucial in the downstream response that they will exert. For example, *ex vivo* pulsing of DCs with oxLDL was shown to reduce atherosclerotic plaque development[181]. In addition, oxLDL-induced apoptosis on DCs has also been found to be atheroprotective[182], suggesting that the plasticity of this cell type can prove useful in immune therapeutics against atherosclerosis. Lastly, chemokine receptor CCR7 on the surface of DCs was decreased in advanced atherosclerotic plaques as compared to control arteries[183], while it was upregulated during atherosclerosis regression[184]. CCR7 is known to drive the migration of cells out of the atherosclerotic plaque and into the lymph nodes, and therefore considered important in regressing plaques[185].

Dendritic cells normally populate lymphoid tissues[186] and are classified as professional antigen presenting cells (APCs)[187]. As such, in atherosclerosis[188], they can degrade the ingested lipid material in their lysosomal compartment, cleave the lipid proteins into smaller peptides and present various peptide fractions on their membranes through major histocompatibility complexes I or II (MHC-I/II)[189,190]. This comprises the initial step for an antigen-specific adaptive immune response. Antigen presentation to adaptive immune cells, primarily T cells, requires three signals in order to take place[191]. Signal I is provided through specific antigen presentation by the MHC-machinery to the T cell receptor[192]. While MHC-I is expressed in all cells, since it is conserved for the fight against viral responses[193], MHC-II is expressed mainly by APCs[187], but also, upon induction, by some non-conventional APCs, like mast cells[187]. The MHC-binding is followed by signal II, which requires the binding of co-stimulatory molecules, such as CD86 or OX40L, to specific T cell costimulatory receptors, like CD28 or OX40 respectively[194]. The fashion of the costimulatory interplay is crucial since it can alter the T cell response from an effector into an inhibitory one[195]. However, there is

still requirement for a third signal, to ensure that the T cell response does not happen in a non-specific manner. Signal III is provided by cytokine molecules[196], usually derived from activated APCs, for example in the form of IL-12, TNFα, or IL-10[197,198]. Similarly to costimulatory interactions, the qualitative and quantitative balance of the local cytokine cocktail can result in the generation of different T cell subsets[199].

Even though both macrophages and DCs present lipid-specific antigens to T cells their main difference was thought to be their migratory capacity. However, this is a notion which is being disputed at recent times[200] and complicates even further the distinction between these two cell types. Nonetheless, in atherosclerosis, DCs can take up antigens at an immature state, travel to distant secondary lymphoid organs, like the spleen and local draining lymph nodes (eg. the heart lymph nodes), whereupon they maturate and present antigens to naïve lymphocytes in the area[201]. Atherosclerosis specific antigenic fragments, that are presented by APCs to lymphocytes and elicit an adaptive immune response, are one of the most unfamiliar territories in the mechanism that shapes atherosclerosis. So far, progress has been made as to identify some of these immunogenic peptides. It has been reported in the past that oxLDL can trigger an autoimmune response through T cell activation[202]. In contrast, apoB100, a particle carried by (V)LDL molecules, has been found to induce a protective adaptive immune response through T cells[203], similarly to heat shock proteins 60/65[204]. This is particularly interesting since it means that specific tolerogenic peptides can be the basis for vaccination strategies against atherosclerosis[205]. In fact, very recently three new peptides of the apoB molecules were discovered to confer protection against the development of atherosclerosis[206], underlining the possibilities that open up towards vaccine development.

3.3 T cells

Naïve T lymphocytes of the $CD4^+$ or $CD8^+$ subset migrate from the thymus and populate peripheral lymphoid tissues, like the spleen[207]. Activation of naïve T cells leads to a subsequent increase in their proliferation rate, which practically means that the T cell receptor is specifically designed to interact with the exact type of antigenic fragment that was presented to their ancestor[208,209]. This process is termed “T cell clonal expansion” and initiates effector and memory responses that will secure immunity upon encounter of the same antigen at a later timepoint. T cells have been generally proven to enhance atherosclerotic plaque development[210,211] and high levels of memory T cells were reported to circulate in atherosclerosis patients[212,213]. Essentially, after getting activated, T cells migrate through the circulation into the atherosclerotic plaque, in order to direct the immune response locally[214].

T cell subset skewing primarily depends on the MHC-machinery, which APCs will use for antigenic fragment presentation. Presentation through MHC-I activates $CD8^+$ T cells[215], while interaction through MHC-II activates $CD4^+$ T cells[216]. This is usually dependent on the means through which APCs have acquired the antigen and the length of the peptide[217].

$CD8^+$ T cells are found at high frequencies in early atherosclerotic plaques, as compared to $CD4^+$ T cells; yet over disease progression, both T cell subsets seem to accumulate and equalize in numbers[218]. $CD8^+$ T cells are mainly suggested to act in a cytotoxic manner, through the secretion of cytokines like interferon-γ (IFNγ)[219] and granzyme B[220] that induce monocyte proliferation and apoptosis respectively. Hence, the role of $CD8^+$ T cells in atherosclerosis is considered detrimental. However, there is accumulating evidence that under certain conditions $CD8^+$ T cells can also act protectively[221]. As in the case of macrophage apoptosis, the stage of plaque development may be a key regulator that defines the protective versus the detrimental contribution of $CD8^+$ T cells in atherosclerosis. Lately it was postulated that a subtype of $CD8^+$ T cells, namely Qa-1 restricted $CD8^+$ T cells, possess regulatory properties[222]. In addition, $CD25^+CD8^+$ T cells were reported to expand in an apoB100 specific manner, which resulted in the secretion of the anti-inflammatory cytokine IL-10[223]. It seems thus that $CD8^+$ T cell subsets in atherosclerosis are now beginning to get explored in more detail.

In contrast, the role of $CD4^+$ T cells in atherosclerosis has been analyzed in more depth. $CD4^+$ T cells can develop into many different subsets, according to the cytokine signals that they receive[224]. For example, T-helper 1 (T_{H1}) and T-helper 2 (T_{H2}) cells differentiate according to the balance of cytokines IL-12/IL-18 and IL-4[225]. Depending on where the cytokine scale leans toward, the transcription factors T-bet or GATA-3 will control the differentiation of a $CD4^+$ T cell into either a T_{H1} or a T_{H2} cell respectively. T_{H1} cells are the most frequent T cell subset in the atherosclerotic plaque[214] and they are proatherogenic[226], mainly through the secretion of cytokine IFNγ[227]. The function of T_{H2} cells however, is not so clearly determined. T_{H2} cells are the main secretors of cytokines IL-4, IL-5 and IL-13; with IL-4[228] reported to counteract the actions of IFNγ[229]. Even though T_{H2} cells have been proven to neutralize the effect of T_{H1} cells[226], they seem to exert both pro-inflammatory and anti-inflammatory effects in atherosclerosis. This is attributed to the quality of their secreted cytokine material. For example, IL-4 was also shown to induce foam cell formation[230] and act in a proatherogenic manner[231]. On the contrary, IL-5 seems to exert a protective effect, by positively affecting the production of athero-protective antibodies by B cells[232]. Another important $CD4^+$ T cell subset is the T_{H17}. These cells arise upon the influence of cytokines IL-6, IL-1β, TGFβ and IL-21, and through activation of transcription factor RORγt, and are considered the main secretors of cytokine IL-17[233]. Their role in atherosclerosis is still unclear, primarily

due to contradictory reports on the effects of IL-17. While a previous study reported reduced plaque formation in the absence of IL-17[234], a follow-up report did not find any effect of IL-17 in atherosclerotic plaque development[235]. Interestingly, IFNγ secretion by T_{H17} cells seems to associate with the progression of coronary atherosclerosis[236], with HDL-particles reported to attenuate T_{H1} and T_{H17} responses[237]. Therefore, there is still a lot to explore on the function of this cell type in atherosclerosis. T_{H17} cells are thought to be the counterparts of the anti-inflammatory $CD4^+$ T_{REG} cell type[238]. The role of T_{REG} cells is essential in atherosclerosis, since they are the main athero-protective cell type[239]. T_{REG} cells are generated through cytokines IL-10 and TGF-β, which activate the transcription factor FoxP3 and lead to the production of the anti-inflammatory cytokine IL-10[240]. The presence of T_{REG} cells is reduced upon atherosclerosis progression[241] and they are negatively associated with the development of myocardial infarction[242]. Their anti-atherogenic manner is attributed mainly to the secretion of IL-10 which can skew macrophages into an M2-phenotype[243] and hamper T_{H1} [244] responses. In fact, T_{REG} cells inhibit inflammatory responses upon atherosclerosis progression while stabilizing plaques during atherosclerosis regression[245]. Thus, T_{REG} cells have become a very attractive target for immune-cell based therapies in atherosclerosis[246].

Finally, there are accumulating reports on additional T cell subsets and their role in cardiovascular syndromes, such as T_{H22} and T_{H9} cells[247]. These cells are mainly defined according to their main secreting cytokine, however, their role is relatively understudied in atherosclerosis. It is important to not forget that even though cytokine secretion profiles are appointed to certain T cell subtypes, this does not mean that these cytokines are cell-type specific. A classic example is cytokine IL-17, which even though it characterizes T_{H17} cells, it has been found to be produced also by other cells like γδ T cells, mast cells and neutrophils[248]. The same can be said for IL-4, which has been linked mainly to T_{H2} cells, yet it can also be secreted by mast cells[249].

Interestingly, even after a T cell has been activated and skewed to a specific subset inside the lymphoid tissues, upon migration within the plaque it may get exposed to differential signals, in terms of quality and quantity. This process can result in a switch of the pre-activated T cell into a different subset[250]. For example, GATA-3 expression on T_{H1} cells can effectively alter them into a T_{H2} phenotype[251]. More specifically for atherosclerosis, it was recently reported that inside progressing plaques, T_{REG} cells acquire a dysfunctional IFNγ secreting phenotype[252]. This indicates how strong the local inflammatory response can be, as to shape *de novo* the T cell response from an anti-inflammatory to a pro-inflammatory one. In the end it is the balance between different cytokines that will shape the net-effect of the immune response inside the plaque area.

3.4 Natural Killer T cells

As mentioned before, antigenic fragments are generally presented through the MHC-I/II molecules. However, there is an additional presentation molecule on the APCs, namely protein CD1d, that is designed to strictly present glycolipid antigens to a distinct population of T cells, termed Natural Killer T (NKT) cells[253]. The NKT cell population migrates from the thymus into the periphery, particularly the spleen and liver, and is reactive to endogenous and exogenous lipid antigens[254]. NKT cells possess a special T cell receptor chain[255] that distinguishes them from other T cell populations and, upon activation, secrete vast amounts of T_{H1}[256] and T_{H2}[257] cytokines. However, they also carry characteristic proteins of the innate immune NK cell population and are therefore considered to act as a bridge between innate and adaptive immunity. Evidently, due to their lipid specific nature, NKT cells are important in atherosclerosis development[258]; particularly during the initial phase[259], when the adaptive response has not yet been established. NKT cells are considered to be proatherogenic[260] due to their potent cytokine secretion, that positively enhances plaque inflammation, particularly through IFNγ[261] and granzyme B[262] secretion. However, they have been found to act also at later stages[263], by affecting atherosclerotic plaque stability through augmentation of local apoptosis. The atherosclerotic ligand presented *via* CD1d to NKT cells is still unknown. Nevertheless, protein MTP, which is involved in hepatic VLDL formation[264], has been found to affect CD1d expression[265], suggesting that there is an endogenous lipid ligand present in atherosclerosis that activates NKT cells[266]. It is important to note that different ligands can affect NKT cells in a way that can be either proinflammatory or anti-inflammatory[260]. In the future, it would be interesting to fully decipher the ligands that activate these cells in atherosclerotic plaques as it would make room for NKT cell specific therapeutic intervention.

3.5 B cells

B lymphocytes are another important adaptive cell type in atherosclerosis[267]. B cells are the antibody producing machinery of the body and in atherosclerosis they have been found to produce both proatherogenic IgG2c[268], as well as, anti-atherogenic IgM[269] antibody fragments. The overall role of B cells in atherosclerosis was initially thought to be protective, since their absence increased plaque development[270]. However, these cells are also distinguished in various subsets with contrasting abilities[271]. Mainly, B cells are separated into B1 cells, which are considered atheroprotective[269,272], and B2 proatherogenic cells[273]. In atherosclerosis, different B cell antibody fragments show different effects. For example, IgM has been found to be indispensable in the protection against atherosclerosis[274], while apoB100-specific IgG fragments are

proatherogenic[275]. Interestingly, a very recent study reported that atherosclerotic mice which lack IgM, show elevation of the IgE antibody fragment, that is specific for mast cell activation[276]. In addition, the GM-CSF producing IRA B cells have also been reported to be proatherogenic[268]. On the contrary, the contribution of IL-10 secreting B cells in atherosclerosis is still somewhat unclear[277] with one study postulating an atheroprotective role[278], while another did not report an effect in atherosclerosis development[279]. B cells reside in large amounts in the spleen and lymph nodes, particularly in the follicular centers and marginal zone[280], but in atherosclerosis they can also be found inside the peritoneal cavity[281], which is rich also in mast cells[282] and foam cells. Their antibody producing capacity in particular, is what makes these cells an obvious target in the development of a vaccination therapy.

3.6 Neutrophils

Another important cell type in atherosclerosis are the neutrophils[283]. This innate granulocytic population, generated in the bone marrow, patrols the blood vessels at high frequencies, and relocates inside the damaged tissue, through signals received by their chemokine receptors[284]. Neutrophils are characterized by their very short lifespan[285] and are rich in chemokine receptors, the most known being CXCR2, and CXCR4[286]. However, under chronic inflammatory conditions, neutrophils can upregulate additional chemokine receptors, such as CCR2 and CXCR3[287]. In atherosclerosis they increase upon hyperlipidemia and accelerate the early phase of plaque development[288]. Neutrophils can crawl on the endothelial cell wall of arterial sites exposed to high shear stress[289], infiltrate the subendothelium[290,291] and inflict local damage, increasing therefore the penetration rate of additional immune cells, like monocytes[292]. They are considered proatherogenic due to their release of proteases and production of MPO[293] and ROS[294], which induce tissue apoptosis. Despite the notion that they only affect early atherogenesis, neutrophils have been reported to act also in end-stage atherosclerosis, mainly through the secretion of matrix metalloproteinases[295]. In support of their role during end-stage atherosclerosis, neutrophils are positively associated with acute coronary events[296]. Interestingly, one manner through which they affect advanced atherosclerosis involves CXCR4; however, this CXCR4 mediated effect of neutrophils was surprisingly found to be protective instead of damaging[297]. In addition, neutrophils possess the unique ability to “explode” under inflammatory conditions, resulting in the formation of what is called neutrophil extracellular traps (NETs)[298]. The presence of NETs in atherosclerosis was described relatively recently[299,300] and, as expected, this was reported to be proatherogenic[301]. Lastly, neutrophil infiltration can also be regulated by other immune cells, such as the mast cells that were found to recruit neutrophils inside the plaques through a CXCR2/CXCL1 interaction[302].

3.7 Mast cells

One of the most important granulocytic populations involved in atherosclerosis are the mast cells[303]. Mast cells are present within the arterial wall at low numbers, even under normal conditions, but accumulate in the area upon atherosclerosis progression[304]. There, mast cells get activated and exert their effects by releasing their granular content in the surrounding microenvironment[305,306]. Mast cells are unique cells, characterized by the stem cell factor receptor, c-kit or CD117[307]. Furthermore, mast cells are recognized by their distinct granular mediators, neutral proteases tryptase and chymase, histamine as well as proinflammatory cytokines like IL-6, TNFα and IFNγ[308]. In the circulation mast cells can be found solely as progenitors and, similar to macrophages, their end-stage maturation takes place only within tissues[309]. Mast cells are largely influenced by the microenvironment at their place of residence and are, thus, distinguished in different subtypes (**Figure 4**). Mouse mast cells are defined as either connective tissue (CTMCs) or mucosal mast cells (MMCs), while human mast cells are classified according to their protease content, which is either only tryptase or tryptase as well as chymase[310]. The activation of mast cells can occur *via* multiple ways. The typical pathway involves binding of an antigen sensitized-IgE antibody onto their characteristic Fcε-receptor[311]. However, mast cells have been reported to get activated *via* additional pathways, such as for instance through TLRs[312], complement receptors[313] and neuropeptide receptors[314]; all activation pathways that have been also implicated in atherosclerosis.

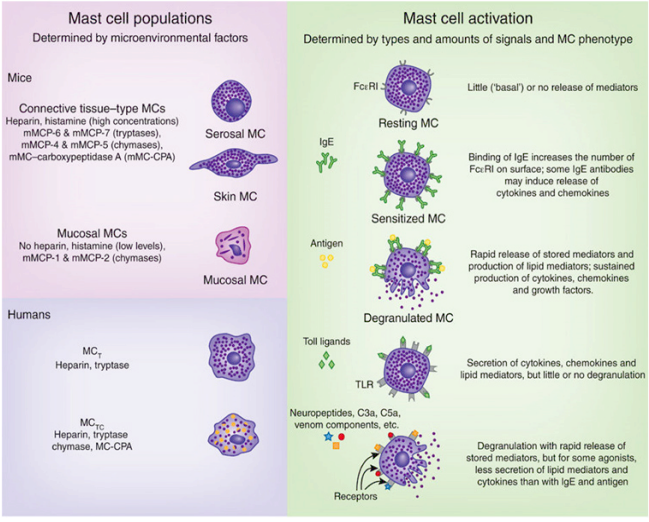


Figure 4. Mast cell subsets and activation pathways. Mast cells are distinguished in various subtypes depending on their place of residence and microenvironment. Mast cell activation can occur through the classical pathway involving binding of an IgE fragment onto their characteristic FcεR, but can also occur though additional pathways. *Adapted from Galli et al., Nat. Immunol. 2011; 12, 1035–1044.*

Mast cells are proatherogenic mediators in atherosclerosis and enhance plaque progression and destabilization[306], through their protease secretion[315] which can degrade the extracellular matrix[316]. They are also reported to enhance foam cell formation, by acting on the cholesterol efflux mechanism[317]. Mast cells possess many chemokine receptors, such as CCR2[318], which can induce their accumulation in the plaque. In addition to their presence inside atherosclerotic plaques, mast cells can also be found in the peritoneal cavity of atherosclerotic mice whereupon mast cell granules have been shown to degrade apoB100[319].

Although mast cells have been investigated thoroughly in atherosclerosis, the exact mast cell activation mechanisms and local behavior within the atherosclerotic plaques is still not fully elucidated. Therefore, it is intriguing to clarify the exact mechanisms that shape mast cells inside the atherosclerotic plaque.

There are many additional cell types that are equally important in atherosclerosis-mediated syndromes which we did not discuss here, such as eosinophils[320], NK cells[321], innate lymphoid cells[322], MDSCs[323], or γδ[324] T cells. Thus, based on the above it is apparent that the immune response in atherosclerosis is a very complex matter. Therefore, a question emerged, on whether atherosclerosis should be classified as a chronic autoimmune disease[325]. In fact, many modern therapeutic interventions target the inflammatory rather than the lipid response. However, we should remember that both mechanisms are co-existing and closely interacting; what's more, they regulate each other in an active manner. An example of this interaction comes from bioactive lipids such as lysophosphatidic acid (LPA). LPA is a lipid component of LDL that can be generated within the plaques upon LDL-modification[326]. LPA has been proven to activate an array of immune cells such as $CD4^+$ T cells[327] and mast cells[328]. In addition, it was only recently reported to enhance foam cell formation, for example through inhibiting SR-BI[329], therefore influencing cholesterol metabolism.

It is thus the intricate interrelation between lipid and immune components that shapes plaque initiation and development and due to that, future research on the mechanisms that target both elements must be the focus of therapeutic means to treat atherosclerosis.

Thesis outline:

The aim of this dissertation has been to investigate the role of mast cells in atherosclerosis, and specifically in immune-mediated pathways that characterize the disease. In **chapter 2** we will give an overview on the role of mast cells in multiple cardiovascular syndromes with a focus on atherosclerosis. **Chapter 3** will provide an example of the interaction between lipid and immune responses in atherosclerosis. We will address the therapeutic capacity offered by blocking the action of lysophosphatidic acid receptors. We will provide evidence on how pharmacological inhibition of the $LPA_{1/3}$ axis can alter the immune response at a systemic level and reduce plaque development. In **chapter 4** we will explore a novel pathway *via* which mast cells can affect atherosclerosis, through acting as non-professional antigen presenting cells. We will provide evidence on how this influences the adaptive immune response towards a proatherogenic manner. In contrast, **chapter 5** will provide evidence on how this capacity of mast cells to present antigens can also elicit a protective response, *via* their CD1d-mediated interaction with NKT cells. We will move onto **chapter 6** to scrutinize the translational impact that mast cells have in atherosclerosis. We will demonstrate how we made use of the flow cytometry method to characterize human intraplaque mast cells and discuss their phenotype in end-stage atherosclerosis. We will conclude the experimental part with **chapter 7** where we will debate the therapeutic ability of mast cells through their role in atherosclerosis regression. Finally, in **chapter 8,** we will summarize the data of this thesis and discuss the latest therapeutic advancements in the field of atherosclerosis.

Reference list:

1. Lederberg, J. Infectious history. *Science* **288,** 287–293 (2000).
2. Plotkin, S. History of vaccination. *Proc. Natl. Acad. Sci. U. S. A.* **111,** 12283–12287 (2014).
3. Arias, E., Heron, M. & Xu, J. United States Life Tables, 2013. *Natl. Vital Stat. Rep.* **66,** 1–64 (2017).
4. DuGoff, E. H., Canudas-Romo, V., Buttorff, C., Leff, B. & Anderson, G. F. Multiple chronic conditions and life expectancy: a life table analysis. *Med. Care* **52,** 688–694 (2014).
5. Benjamin, E. J. *et al.* Heart Disease and Stroke Statistics-2017 Update: A Report From the American Heart Association. *Circulation* **135,** e146–e603 (2017).
6. Dalen, J. E., Alpert, J. S., Goldberg, R. J. & Weinstein, R. S. The epidemic of the 20(th) century: coronary heart disease. *Am. J. Med.* **127,** 807–812 (2014).
7. Fuster, V., Badimon, L., Badimon, J. J. & H.Chesebro, J. The pathogenesis of coronary artery disease and the acute coronary syndromes. *N. Engl. J. Med.* (1992).
8. Thompson, R. C. *et al.* Atherosclerosis across 4000 years of human history: the Horus study of four ancient populations. *Lancet (London, England)* **381,** 1211–1222 (2013).
9. Torres, N., Guevara-Cruz, M., Velazquez-Villegas, L. A. & Tovar, A. R. Nutrition and Atherosclerosis. *Arch. Med. Res.* **46,** 408–426 (2015).
10. World Health Organization. WHO - The top 10 causes of death. Available at: http://www.who.int/mediacentre/factsheets/fs310/en/.
11. Konstantinov, I. E. & Jankovic, G. M. Alexander I. Ignatowski: a pioneer in the study of atherosclerosis. *Texas Hear. Inst. J.* **40,** 246–249 (2013).
12. Buja, L. M. Nikolai N. Anitschkow and the lipid hypothesis of atherosclerosis. *Cardiovasc. Pathol.* **23,** 183–184 (2014).
13. Gerrity, R. G. The role of the monocyte in atherogenesis: I. Transition of blood-borne monocytes into foam cells in fatty lesions. *Am. J. Pathol.* **103,** 181–190 (1981).
14. Klurfeld, D. M. Interactions of immune function with lipids and atherosclerosis. *Crit. Rev. Toxicol.* **11,** 333–365 (1983).
15. Ross, R. The pathogenesis of atherosclerosis--an update. *N. Engl. J. Med.* **314,** 488–500 (1986).
16. Usman, A., Ribatti, D., Sadat, U. & Gillard, J. H. From Lipid Retention to Immune-Mediate Inflammation and Associated Angiogenesis in the Pathogenesis of Atherosclerosis. *J. Atheroscler. Thromb.* **22,** 739–749 (2015).
17. Legein, B., Temmerman, L., Biessen, E. A. L. & Lutgens, E. Inflammation and immune system interactions in atherosclerosis. *Cell. Mol. Life Sci.* **70,** 3847–3869 (2013).
18. Insull, W. J. The pathology of atherosclerosis: plaque development and plaque responses to medical treatment. *Am. J. Med.* **122,** S3–S14 (2009).
19. Bentzon, J. F., Otsuka, F., Virmani, R. & Falk, E. Mechanisms of plaque formation and rupture. *Circ. Res.* **114,** 1852–1866 (2014).
20. Li, X., Yang, Q., Wang, Z. & Wei, D. Shear stress in atherosclerotic plaque determination. *DNA Cell Biol.* **33,** 830–838 (2014).
21. Ando, J. & Yamamoto, K. Flow detection and calcium signalling in vascular endothelial cells. *Cardiovasc. Res.* **99,** 260–268 (2013).
22. Havel, R. J. Biology of cholesterol, lipoproteins and atherosclerosis. *Clin. Exp. Hypertens. A.* **11,** 887–900 (1989).
23. Kruth, H. S. Lipoprotein cholesterol and atherosclerosis. *Curr. Mol. Med.* **1,** 633–653 (2001).
24. Won, D. *et al.* Relative reduction of endothelial nitric-oxide synthase expression and transcription in atherosclerosis-prone regions of the mouse aorta and in an in vitro

model of disturbed flow. *Am. J. Pathol.* **171,** 1691–1704 (2007).

25. Hirase, T. & Node, K. Endothelial dysfunction as a cellular mechanism for vascular failure. *Am. J. Physiol. Heart Circ. Physiol.* **302,** H499-505 (2012).
26. Conway, D. E. *et al.* Fluid shear stress on endothelial cells modulates mechanical tension across VE-cadherin and PECAM-1. *Curr. Biol.* **23,** 1024–1030 (2013).
27. Davies, M. J. *et al.* The expression of the adhesion molecules ICAM-1, VCAM-1, PECAM, and E-selectin in human atherosclerosis. *J. Pathol.* **171,** 223–229 (1993).
28. Kang, H., Cancel, L. M. & Tarbell, J. M. Effect of shear stress on water and LDL transport through cultured endothelial cell monolayers. *Atherosclerosis* **233,** 682–690 (2014).
29. Boren, J. *et al.* Identification of the principal proteoglycan-binding site in LDL. A single-point mutation in apo-B100 severely affects proteoglycan interaction without affecting LDL receptor binding. *J. Clin. Invest.* **101,** 2658–2664 (1998).
30. Bancells, C. *et al.* High binding affinity of electronegative LDL to human aortic proteoglycans depends on its aggregation level. *J. Lipid Res.* **50,** 446–455 (2009).
31. Alique, M., Luna, C., Carracedo, J. & Ramirez, R. LDL biochemical modifications: a link between atherosclerosis and aging. *Food Nutr. Res.* **59,** 29240 (2015).
32. Chistiakov, D. A., Orekhov, A. N. & Bobryshev, Y. V. Endothelial PECAM-1 and its function in vascular physiology and atherogenic pathology. *Exp. Mol. Pathol.* **100,** 409–415 (2016).
33. Schmitt, M. M. N. *et al.* Endothelial junctional adhesion molecule-a guides monocytes into flow-dependent predilection sites of atherosclerosis. *Circulation* **129,** 66–76 (2014).
34. Braunersreuther, V., Mach, F. & Steffens, S. The specific role of chemokines in atherosclerosis. *Thromb. Haemost.* **97,** 714–721 (2007).
35. Rosenfeld, M. E. An overview of the evolution of the atherosclerotic plaque: from fatty streak to plaque rupture and thrombosis. *Z. Kardiol.* **89 Suppl 7,** 2–6 (2000).
36. Stary, H. C. Lipid and macrophage accumulations in arteries of children and the development of atherosclerosis. *Am. J. Clin. Nutr.* **72,** 1297S–1306S (2000).
37. Bjorkegren, J. L. M. *et al.* Plasma cholesterol-induced lesion networks activated before regression of early, mature, and advanced atherosclerosis. *PLoS Genet.* **10,** e1004201 (2014).
38. Silvestre-Roig, C. *et al.* Atherosclerotic plaque destabilization: mechanisms, models, and therapeutic strategies. *Circ. Res.* **114,** 214–226 (2014).
39. Saxena, A., McMeekin, J. D. & Thomson, D. J. Expression of Bcl-x, Bcl-2, Bax, and Bak in endarterectomy and atherectomy specimens. *J. Pathol.* **196,** 335–342 (2002).
40. Gautier, E. L. *et al.* Macrophage apoptosis exerts divergent effects on atherogenesis as a function of lesion stage. *Circulation* **119,** 1795–1804 (2009).
41. Van Vre, E. A., Ait-Oufella, H., Tedgui, A. & Mallat, Z. Apoptotic cell death and efferocytosis in atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* **32,** 887–893 (2012).
42. Lin, J. *et al.* A role of RIP3-mediated macrophage necrosis in atherosclerosis development. *Cell Rep.* **3,** 200–210 (2013).
43. Tabas, I. Macrophage death and defective inflammation resolution in atherosclerosis. *Nat. Rev. Immunol.* **10,** 36–46 (2010).
44. Gonzalez, L. & Trigatti, B. L. Macrophage Apoptosis and Necrotic Core Development in Atherosclerosis: A Rapidly Advancing Field with Clinical Relevance to Imaging and Therapy. *Can. J. Cardiol.* **33,** 303–312 (2017).
45. Han, S. *et al.* Macrophage insulin receptor deficiency increases ER stress-induced apoptosis and necrotic core formation in advanced atherosclerotic lesions. *Cell Metab.* **3,** 257–266 (2006).
46. Bennett, M. R., Sinha, S. & Owens, G. K. Vascular Smooth Muscle Cells in Atherosclerosis. *Circ. Res.* **118,** 692–702 (2016).

47. Shami, A., Goncalves, I. & Hultgardh-Nilsson, A. Collagen and related extracellular matrix proteins in atherosclerotic plaque development. *Curr. Opin. Lipidol.* **25,** 394–399 (2014).
48. van Hinsbergh, V. W. M., Eringa, E. C. & Daemen, M. J. A. P. Neovascularization of the atherosclerotic plaque: interplay between atherosclerotic lesion, adventitia-derived microvessels and perivascular fat. *Curr. Opin. Lipidol.* **26,** 405–411 (2015).
49. Cherepanova, O. A. *et al.* Oxidized phospholipids induce type VIII collagen expression and vascular smooth muscle cell migration. *Circ. Res.* **104,** 609–618 (2009).
50. Plenz, G. A. M., Deng, M. C., Robenek, H. & Volker, W. Vascular collagens: spotlight on the role of type VIII collagen in atherogenesis. *Atherosclerosis* **166,** 1–11 (2003).
51. Newby, A. C. & Zaltsman, A. B. Fibrous cap formation or destruction--the critical importance of vascular smooth muscle cell proliferation, migration and matrix formation. *Cardiovasc. Res.* **41,** 345–360 (1999).
52. Lopes, J. *et al.* Type VIII collagen mediates vessel wall remodeling after arterial injury and fibrous cap formation in atherosclerosis. *Am. J. Pathol.* **182,** 2241–2253 (2013).
53. Sluimer, J. C. & Daemen, M. J. Novel concepts in atherogenesis: angiogenesis and hypoxia in atherosclerosis. *J. Pathol.* **218,** 7–29 (2009).
54. Kumamoto, M., Nakashima, Y. & Sueishi, K. Intimal neovascularization in human coronary atherosclerosis: Its origin and pathophysiological significance. *Hum. Pathol.* **26,** 450–456 (1995).
55. Virmani, R. *et al.* Atherosclerotic plaque progression and vulnerability to rupture: Angiogenesis as a source of intraplaque hemorrhage. *Arteriosclerosis, Thrombosis, and Vascular Biology* **25,** 2054–2061 (2005).
56. Virmani, R., Burke, A. P., Farb, A. & Kolodgie, F. D. Pathology of the vulnerable plaque. *J. Am. Coll. Cardiol.* **47,** C13-8 (2006).
57. Otsuka, F. *et al.* Natural progression of atherosclerosis from pathologic intimal thickening to late fibroatheroma in human coronary arteries: A pathology study. *Atherosclerosis* **241,** 772–782 (2015).
58. Badimon, L. & Vilahur, G. Thrombosis formation on atherosclerotic lesions and plaque rupture. *J. Intern. Med.* **276,** 618–632 (2014).
59. White, S. J., Newby, A. C. & Johnson, T. W. Endothelial erosion of plaques as a substrate for coronary thrombosis. *Thromb. Haemost.* **115,** 509–519 (2016).
60. Ahmadi, A. *et al.* Do plaques rapidly progress prior to myocardial infarction? The interplay between plaque vulnerability and progression. *Circ. Res.* **117,** 99–104 (2015).
61. Niesten, J. M. *et al.* Relationship between thrombus attenuation and different stroke subtypes. *Neuroradiology* **55,** 1071–1079 (2013).
62. Apinis, A., Sehgal, S. & Leff, J. Intraoperative management of carotid endarterectomy. *Anesthesiol. Clin.* **32,** 677–698 (2014).
63. Ju, M. H. & Rodriguez, H. E. Standard balloon angioplasty versus angioplasty with paclitaxel-eluting balloons for femoropopliteal artery stenosis. *J. Cardiovasc. Surg. (Torino).* **53,** 459–463 (2012).
64. Phatouros, C. C. *et al.* Carotid artery stent placement for atherosclerotic disease: rationale, technique, and current status. *Radiology* **217,** 26–41 (2000).
65. Kulik, A. & Ruel, M. Lipid-lowering therapy and coronary artery bypass graft surgery: what are the benefits? *Curr. Opin. Cardiol.* **26,** 508–517 (2011).
66. Kim, M. S. & Dean, L. S. In-Stent Restenosis. *Cardiovascular Therapeutics* **29,** 190–198 (2011).
67. Libby, P. & Aikawa, M. Mechanisms of plaque stabilization with statins. *Am. J. Cardiol.* **91,** 4B–8B (2003).
68. Koch, C. G. Statin therapy. *Curr. Pharm. Des.* **18,** 6284–6290 (2012).

69. Nissen, S. E. *et al.* Effect of very high-intensity statin therapy on regression of coronary atherosclerosis: the ASTEROID trial. *JAMA* **295,** 1556–1565 (2006).
70. van Lammeren, G. W. *et al.* Time-dependent changes in atherosclerotic plaque composition in patients undergoing carotid surgery. *Circulation* **129,** 2269–2276 (2014).
71. Libby, P. & Pasterkamp, G. Requiem for the 'vulnerable plaque'. *Eur. Heart J.* **36,** 2984–2987 (2015).
72. Kanter, J. E. & Bornfeldt, K. E. Inflammation and diabetes-accelerated atherosclerosis: myeloid cell mediators. *Trends Endocrinol. Metab.* **24,** 137–144 (2013).
73. Hurtubise, J. *et al.* The Different Facets of Dyslipidemia and Hypertension in Atherosclerosis. *Curr. Atheroscler. Rep.* **18,** 82 (2016).
74. Siasos, G. *et al.* Smoking and atherosclerosis: mechanisms of disease and new therapeutic approaches. *Curr. Med. Chem.* **21,** 3936–3948 (2014).
75. Mathur, P., Ostadal, B., Romeo, F. & Mehta, J. L. Gender-Related Differences in Atherosclerosis. *Cardiovasc. drugs Ther.* **29,** 319–327 (2015).
76. Getz, G. S. & Reardon, C. A. Animal models of atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* **32,** 1104–1115 (2012).
77. Brousseau, M. E. & Hoeg, J. M. Transgenic rabbits as models for atherosclerosis research. *J. Lipid Res.* **40,** 365–375 (1999).
78. Mott, G. E., Jackson, E. M., McMahan, C. A. & McGill, H. C. J. Dietary cholesterol and type of fat differentially affect cholesterol metabolism and atherosclerosis in baboons. *J. Nutr.* **122,** 1397–1406 (1992).
79. Bentzon, J. F. & Falk, E. Atherosclerotic lesions in mouse and man: is it the same disease? *Curr. Opin. Lipidol.* **21,** 434–440 (2010).
80. Haitas, B., Baker, S. G., Meyer, T. E., Joffe, B. I. & Seftel, H. C. Natural history and cardiac manifestations of homozygous familial hypercholesterolaemia. *Q. J. Med.* **76,** 731–740 (1990).
81. Magallon, J., Egalka, M. & Diacovo, T. G. Humanizing thrombi in mice. *Trends Cardiovasc. Med.* **21,** 33–36 (2011).
82. Miller, W. L. & Bose, H. S. Early steps in steroidogenesis: intracellular cholesterol trafficking. *J. Lipid Res.* **52,** 2111–2135 (2011).
83. Norlin, M. & Wikvall, K. Enzymes in the conversion of cholesterol into bile acids. *Curr. Mol. Med.* **7,** 199–218 (2007).
84. Rog, T. & Vattulainen, I. Cholesterol, sphingolipids, and glycolipids: what do we know about their role in raft-like membranes? *Chem. Phys. Lipids* **184,** 82–104 (2014).
85. Dalen, J. E. & Devries, S. Diets to prevent coronary heart disease 1957-2013: what have we learned? *Am. J. Med.* **127,** 364–369 (2014).
86. Kruit, J.-K., Groen, A. K., van Berkel, T. J. & Kuipers, F. Emerging roles of the intestine in control of cholesterol metabolism. *World J. Gastroenterol.* **12,** 6429–6439 (2006).
87. Dietschy, J. M., Turley, S. D. & Spady, D. K. Role of liver in the maintenance of cholesterol and low density lipoprotein homeostasis in different animal species, including humans. *J. Lipid Res.* **34,** 1637–1659 (1993).
88. Gylling, H. Cholesterol metabolism and its implications for therapeutic interventions in patients with hypercholesterolaemia. *Int. J. Clin. Pract.* **58,** 859–866 (2004).
89. Horton, J. D., Goldstein, J. L. & Brown, M. S. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J. Clin. Invest.* **109,** 1125–1131 (2002).
90. Ness, G. C. Physiological feedback regulation of cholesterol biosynthesis: Role of translational control of hepatic HMG-CoA reductase and possible involvement of oxylanosterols. *Biochim. Biophys. Acta* **1851,** 667–673 (2015).
91. Ramasamy, I. Recent advances in physiological lipoprotein metabolism. *Clin. Chem. Lab.*

Med. **52,** 1695–1727 (2014).

92. Go, G.-W. & Mani, A. Low-density lipoprotein receptor (LDLR) family orchestrates cholesterol homeostasis. *Yale J. Biol. Med.* **85,** 19–28 (2012).
93. Chiang, J. Y. Regulation of bile acid synthesis. *Front. Biosci.* **3,** d176-93 (1998).
94. Hong, C. & Tontonoz, P. Liver X receptors in lipid metabolism: opportunities for drug discovery. *Nat. Rev. Drug Discov.* **13,** 433–444 (2014).
95. Zelcer, N., Hong, C., Boyadjian, R. & Tontonoz, P. LXR regulates cholesterol uptake through Idol-dependent ubiquitination of the LDL receptor. *Science* **325,** 100–104 (2009).
96. Repa, J. J. *et al.* Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRalpha and LXRbeta. *Genes Dev.* **14,** 2819–2830 (2000).
97. Hussein, M. A. *et al.* LXR-Mediated ABCA1 Expression and Function Are Modulated by High Glucose and PRMT2. *PLoS One* **10,** e0135218 (2015).
98. Wang, N., Ranalletta, M., Matsuura, F., Peng, F. & Tall, A. R. LXR-induced redistribution of ABCG1 to plasma membrane in macrophages enhances cholesterol mass efflux to HDL. *Arterioscler. Thromb. Vasc. Biol.* **26,** 1310–1316 (2006).
99. Ji, A. *et al.* Nascent HDL formation in hepatocytes and role of ABCA1, ABCG1, and SR-BI. *J. Lipid Res.* **53,** 446–455 (2012).
100. Kennedy, M. A. *et al.* ABCG1 has a critical role in mediating cholesterol efflux to HDL and preventing cellular lipid accumulation. *Cell Metab.* **1,** 121–131 (2005).
101. Zannis, V. I. *et al.* HDL biogenesis, remodeling, and catabolism. *Handb. Exp. Pharmacol.* **224,** 53–111 (2015).
102. Trigatti, B. L. & Fuller, M. HDL signaling and protection against coronary artery atherosclerosis in mice. *J. Biomed. Res.* **30,** 94–100 (2016).
103. Gordon, T., Castelli, W. P., Hjortland, M. C., Kannel, W. B. & Dawber, T. R. High density lipoprotein as a protective factor against coronary heart disease. The Framingham Study. *Am. J. Med.* **62,** 707–714 (1977).
104. Kypreos, K. E. & Zannis, V. I. Pathway of biogenesis of apolipoprotein E-containing HDL in vivo with the participation of ABCA1 and LCAT. *Biochem. J.* **403,** 359–367 (2007).
105. Badimon, J. J., Badimon, L. & Fuster, V. Regression of atherosclerotic lesions by high density lipoprotein plasma fraction in the cholesterol-fed rabbit. *J. Clin. Invest.* **85,** 1234–1241 (1990).
106. Feig, J. E. *et al.* HDL promotes rapid atherosclerosis regression in mice and alters inflammatory properties of plaque monocyte-derived cells. *Proc. Natl. Acad. Sci. U. S. A.* **108,** 7166–7171 (2011).
107. Rubin, E. M., Krauss, R. M., Spangler, E. A., Verstuyft, J. G. & Clift, S. M. Inhibition of early atherogenesis in transgenic mice by human apolipoprotein AI. *Nature* **353,** 265–267 (1991).
108. Liu, J. *et al.* Non-high-density lipoprotein and very-low-density lipoprotein cholesterol and their risk predictive values in coronary heart disease. *Am. J. Cardiol.* **98,** 1363–1368 (2006).
109. Chen, Z. *et al.* Serum cholesterol concentration and coronary heart disease in population with low cholesterol concentrations. *BMJ* **303,** 276–282 (1991).
110. Gould, A. L., Davies, G. M., Alemao, E., Yin, D. D. & Cook, J. R. Cholesterol reduction yields clinical benefits: meta-analysis including recent trials. *Clin. Ther.* **29,** 778–794 (2007).
111. Baigent, C. *et al.* Efficacy and safety of more intensive lowering of LDL cholesterol: a meta-analysis of data from 170,000 participants in 26 randomised trials. *Lancet (London, England)* **376,** 1670–1681 (2010).
112. Holvoet, P., Theilmeier, G., Shivalkar, B., Flameng, W. & Collen, D. LDL hypercholesterolemia

is associated with accumulation of oxidized LDL, atherosclerotic plaque growth, and compensatory vessel enlargement in coronary arteries of miniature pigs. *Arterioscler. Thromb. Vasc. Biol.* **18,** 415–422 (1998).

113. Shashkin, P., Dragulev, B. & Ley, K. Macrophage differentiation to foam cells. *Curr. Pharm. Des.* **11,** 3061–3072 (2005).
114. Park, Y. M. CD36, a scavenger receptor implicated in atherosclerosis. *Exp. Mol. Med.* **46,** e99 (2014).
115. Brown, M. S., Goldstein, J. L., Krieger, M., Ho, Y. K. & Anderson, R. G. Reversible accumulation of cholesteryl esters in macrophages incubated with acetylated lipoproteins. *J. Cell Biol.* **82,** 597–613 (1979).
116. Ouimet, M. & Marcel, Y. L. Regulation of lipid droplet cholesterol efflux from macrophage foam cells. *Arterioscler. Thromb. Vasc. Biol.* **32,** 575–581 (2012).
117. Cavelier, C., Lorenzi, I., Rohrer, L. & von Eckardstein, A. Lipid efflux by the ATP-binding cassette transporters ABCA1 and ABCG1. *Biochim. Biophys. Acta* **1761,** 655–666 (2006).
118. Cuchel, M. & Rader, D. J. Macrophage reverse cholesterol transport: key to the regression of atherosclerosis? *Circulation* **113,** 2548–2555 (2006).
119. Favari, E. *et al.* Cholesterol efflux and reverse cholesterol transport. *Handb. Exp. Pharmacol.* **224,** 181–206 (2015).
120. Acton, S. *et al.* Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. *Science* **271,** 518–520 (1996).
121. Ji, Y. *et al.* Hepatic scavenger receptor BI promotes rapid clearance of high density lipoprotein free cholesterol and its transport into bile. *J. Biol. Chem.* **274,** 33398–33402 (1999).
122. Tabas, I. Apoptosis and plaque destabilization in atherosclerosis: the role of macrophage apoptosis induced by cholesterol. *Cell death and differentiation* **11 Suppl 1,** S12-6 (2004).
123. Zhang, S. H., Reddick, R. L., Piedrahita, J. A. & Maeda, N. Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. *Science* **258,** 468–471 (1992).
124. Ishibashi, S. *et al.* Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery. *J. Clin. Invest.* **92,** 883–893 (1993).
125. Getz, G. S. & Reardon, C. A. Do the Apoe-/- and Ldlr-/- Mice Yield the Same Insight on Atherogenesis? *Arteriosclerosis, thrombosis, and vascular biology* (2016). doi:10.1161/ATVBAHA.116.306874
126. Daugherty, A. *et al.* Recommendation on Design, Execution, and Reporting of Animal Atherosclerosis Studies: A Scientific Statement From the American Heart Association. *Circ. Res.* (2017). doi:10.1161/RES.0000000000000169
127. Weingartner, O., Lutjohann, D., Bohm, M. & Laufs, U. Relationship between cholesterol synthesis and intestinal absorption is associated with cardiovascular risk. *Atherosclerosis* **210,** 362–365 (2010).
128. Gistera, A. & Hansson, G. K. The immunology of atherosclerosis. *Nat. Rev. Nephrol.* **13,** 368–380 (2017).
129. Chavez-Sanchez, L. *et al.* Innate immune system cells in atherosclerosis. *Arch. Med. Res.* **45,** 1–14 (2014).
130. Ketelhuth, D. F. J. & Hansson, G. K. Adaptive Response of T and B Cells in Atherosclerosis. *Circ. Res.* **118,** 668–678 (2016).
131. Zarzycka, B., Nicolaes, G. A. F. & Lutgens, E. Targeting the adaptive immune system: new strategies in the treatment of atherosclerosis. *Expert Rev. Clin. Pharmacol.* **8,** 297–313 (2015).
132. Zhang, M. *et al.* AMP-activated protein kinase alpha1 promotes atherogenesis by

increasing monocyte-to-macrophage differentiation. *J. Biol. Chem.* **292,** 7888–7903 (2017).

133. Ley, K., Miller, Y. I. & Hedrick, C. C. Monocyte and macrophage dynamics during atherogenesis. *Arterioscler. Thromb. Vasc. Biol.* **31,** 1506–1516 (2011).

134. Yona, S. & Jung, S. Monocytes: subsets, origins, fates and functions. *Curr. Opin. Hematol.* **17,** 53–59 (2010).

135. Gerhardt, T. & Ley, K. Monocyte trafficking across the vessel wall. *Cardiovasc. Res.* **107,** 321–330 (2015).

136. Mestas, J. & Ley, K. Monocyte-endothelial cell interactions in the development of atherosclerosis. *Trends Cardiovasc. Med.* **18,** 228–232 (2008).

137. Drechsler, M., Duchene, J. & Soehnlein, O. Chemokines control mobilization, recruitment, and fate of monocytes in atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* **35,** 1050–1055 (2015).

138. Deshmane, S. L., Kremlev, S., Amini, S. & Sawaya, B. E. Monocyte chemoattractant protein-1 (MCP-1): an overview. *J. Interferon Cytokine Res.* **29,** 313–326 (2009).

139. Boring, L., Gosling, J., Cleary, M. & Charo, I. F. Decreased lesion formation in CCR2-/- mice reveals a role for chemokines in the initiation of atherosclerosis. *Nature* **394,** 894–897 (1998).

140. Coll, B., Alonso-Villaverde, C. & Joven, J. Monocyte chemoattractant protein-1 and atherosclerosis: is there room for an additional biomarker? *Clin. Chim. Acta.* **383,** 21–29 (2007).

141. Wong, K. L. *et al.* The three human monocyte subsets: implications for health and disease. *Immunol. Res.* **53,** 41–57 (2012).

142. Rogacev, K. S. *et al.* CD14++CD16+ monocytes independently predict cardiovascular events: a cohort study of 951 patients referred for elective coronary angiography. *J. Am. Coll. Cardiol.* **60,** 1512–1520 (2012).

143. Sunderkotter, C. *et al.* Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response. *J. Immunol.* **172,** 4410–4417 (2004).

144. Hanna, R. N. *et al.* NR4A1 (Nur77) deletion polarizes macrophages toward an inflammatory phenotype and increases atherosclerosis. *Circ. Res.* **110,** 416–427 (2012).

145. Hamers, A. A. J. *et al.* Bone marrow-specific deficiency of nuclear receptor Nur77 enhances atherosclerosis. *Circ. Res.* **110,** 428–438 (2012).

146. Swirski, F. K. *et al.* Ly-6Chi monocytes dominate hypercholesterolemia-associated monocytosis and give rise to macrophages in atheromata. *J. Clin. Invest.* **117,** 195–205 (2007).

147. Bastiaansen, A. J. N. M. *et al.* TLR4 accessory molecule RP105 (CD180) regulates monocyte-driven arteriogenesis in a murine hind limb ischemia model. *PLoS One* **9,** e99882 (2014).

148. Wezel, A. *et al.* RP105 deficiency attenuates early atherosclerosis via decreased monocyte influx in a CCR2 dependent manner. *Atherosclerosis* **238,** 132–139 (2015).

149. Williams, H. J., Fisher, E. A. & Greaves, D. R. Macrophage differentiation and function in atherosclerosis: opportunities for therapeutic intervention? *J. Innate Immun.* **4,** 498–508 (2012).

150. Fu, Y. *et al.* Caveolin-1 plays a critical role in the differentiation of monocytes into macrophages. *Arterioscler. Thromb. Vasc. Biol.* **32,** e117-25 (2012).

151. Kushiyama, A. *et al.* Xanthine oxidoreductase is involved in macrophage foam cell formation and atherosclerosis development. *Arterioscler. Thromb. Vasc. Biol.* **32,** 291–298 (2012).

152. Ishihara, T. *et al.* Foamy cells associated with phagocytosis of glutaraldehyde-treated red

blood cells and red cell membranes. *Acta Pathol. Jpn.* **37,** 627–637 (1987).

153. Park, H., Ishihara, D. & Cox, D. Regulation of tyrosine phosphorylation in macrophage phagocytosis and chemotaxis. *Arch. Biochem. Biophys.* **510,** 101–111 (2011).
154. Chen, Y.-J. *et al.* Eps8 protein facilitates phagocytosis by increasing TLR4-MyD88 protein interaction in lipopolysaccharide-stimulated macrophages. *J. Biol. Chem.* **287,** 18806–18819 (2012).
155. Geng, H. *et al.* The effects of ox-LDL in human atherosclerosis may be mediated in part via the toll-like receptor 4 pathway. *Mol. Cell. Biochem.* **342,** 201–206 (2010).
156. Chavez-Sanchez, L. *et al.* The role of TLR2, TLR4 and CD36 in macrophage activation and foam cell formation in response to oxLDL in humans. *Hum. Immunol.* **75,** 322–329 (2014).
157. Blich, M. *et al.* Macrophage activation by heparanase is mediated by TLR-2 and TLR-4 and associates with plaque progression. *Arterioscler. Thromb. Vasc. Biol.* **33,** e56-65 (2013).
158. Seimon, T. A. *et al.* Atherogenic lipids and lipoproteins trigger CD36-TLR2-dependent apoptosis in macrophages undergoing endoplasmic reticulum stress. *Cell Metab.* **12,** 467–482 (2010).
159. Gautier, E. L. *et al.* Macrophage apoptosis exerts divergent effects on atherogenesis as a function of lesion stage. *Circulation* **119,** 1795–1804 (2009).
160. Schrijvers, D. M., De Meyer, G. R. Y., Herman, A. G. & Martinet, W. Phagocytosis in atherosclerosis: Molecular mechanisms and implications for plaque progression and stability. *Cardiovasc. Res.* **73,** 470–480 (2007).
161. Chinetti-Gbaguidi, G., Colin, S. & Staels, B. Macrophage subsets in atherosclerosis. *Nat. Rev. Cardiol.* **12,** 10–17 (2015).
162. Mills, C. D. Anatomy of a discovery: m1 and m2 macrophages. *Front. Immunol.* **6,** 212 (2015).
163. Colin, S., Chinetti-Gbaguidi, G. & Staels, B. Macrophage phenotypes in atherosclerosis. *Immunol. Rev.* **262,** 153–166 (2014).
164. Cho, K. Y. *et al.* The phenotype of infiltrating macrophages influences arteriosclerotic plaque vulnerability in the carotid artery. *J. Stroke Cerebrovasc. Dis.* **22,** 910–918 (2013).
165. Mehta, N. U. *et al.* Apolipoprotein E-/- Mice Lacking Hemopexin Develop Increased Atherosclerosis via Mechanisms That Include Oxidative Stress and Altered Macrophage Function. *Arterioscler. Thromb. Vasc. Biol.* **36,** 1152–1163 (2016).
166. Rahman, K. *et al.* Inflammatory Ly6Chi monocytes and their conversion to M2 macrophages drive atherosclerosis regression. *J. Clin. Invest.* **127,** 2904–2915 (2017).
167. Kadl, A. *et al.* Identification of a novel macrophage phenotype that develops in response to atherogenic phospholipids via Nrf2. *Circ. Res.* **107,** 737–746 (2010).
168. De Paoli, F., Staels, B. & Chinetti-Gbaguidi, G. Macrophage phenotypes and their modulation in atherosclerosis. *Circ. J.* **78,** 1775–1781 (2014).
169. Geissmann, F. *et al.* Development of monocytes, macrophages, and dendritic cells. *Science* **327,** 656–661 (2010).
170. Lindquist, R. L. *et al.* Visualizing dendritic cell networks in vivo. *Nat. Immunol.* **5,** 1243–1250 (2004).
171. Hume, D. A., Robinson, A. P., MacPherson, G. G. & Gordon, S. The mononuclear phagocyte system of the mouse defined by immunohistochemical localization of antigen F4/80. Relationship between macrophages, Langerhans cells, reticular cells, and dendritic cells in lymphoid and hematopoietic organs. *J. Exp. Med.* **158,** 1522–1536 (1983).
172. Probst, H. C. *et al.* Histological analysis of CD11c-DTR/GFP mice after in vivo depletion of dendritic cells. *Clin. Exp. Immunol.* **141,** 398–404 (2005).
173. Wu, H. *et al.* Functional role of CD11c+ monocytes in atherogenesis associated with

hypercholesterolemia. *Circulation* **119,** 2708–2717 (2009).

174. Paulson, K. E. *et al.* Resident intimal dendritic cells accumulate lipid and contribute to the initiation of atherosclerosis. *Circ. Res.* **106,** 383–390 (2010).
175. Zernecke, A. Dendritic cells in atherosclerosis: evidence in mice and humans. *Arterioscler. Thromb. Vasc. Biol.* **35,** 763–770 (2015).
176. Allahverdian, S., Chehroudi, A. C., McManus, B. M., Abraham, T. & Francis, G. A. Contribution of intimal smooth muscle cells to cholesterol accumulation and macrophage-like cells in human atherosclerosis. *Circulation* **129,** 1551–1559 (2014).
177. Taghavie-Moghadam, P. L., Butcher, M. J. & Galkina, E. V. The dynamic lives of macrophage and dendritic cell subsets in atherosclerosis. *Ann. N. Y. Acad. Sci.* **1319,** 19–37 (2014).
178. Choi, J.-H. *et al.* Flt3 signaling-dependent dendritic cells protect against atherosclerosis. *Immunity* **35,** 819–831 (2011).
179. Doring, Y. *et al.* Auto-antigenic protein-DNA complexes stimulate plasmacytoid dendritic cells to promote atherosclerosis. *Circulation* **125,** 1673–1683 (2012).
180. Nickel, T. *et al.* oxLDL uptake by dendritic cells induces upregulation of scavenger-receptors, maturation and differentiation. *Atherosclerosis* **205,** 442–450 (2009).
181. Habets, K. L. L. *et al.* Vaccination using oxidized low-density lipoprotein-pulsed dendritic cells reduces atherosclerosis in LDL receptor-deficient mice. *Cardiovasc. Res.* **85,** 622–630 (2010).
182. Frodermann, V. *et al.* Oxidized low-density lipoprotein-induced apoptotic dendritic cells as a novel therapy for atherosclerosis. *J. Immunol.* **194,** 2208–2218 (2015).
183. Nickel, T. *et al.* oxLDL downregulates the dendritic cell homing factors CCR7 and CCL21. *Mediators Inflamm.* **2012,** 320953 (2012).
184. Trogan, E. *et al.* Gene expression changes in foam cells and the role of chemokine receptor CCR7 during atherosclerosis regression in ApoE-deficient mice. *Proc. Natl. Acad. Sci. U. S. A.* **103,** 3781–3786 (2006).
185. Feig, J. E. *et al.* Statins promote the regression of atherosclerosis via activation of the CCR7-dependent emigration pathway in macrophages. *PLoS One* **6,** e28534 (2011).
186. Naik, S. H. *et al.* Intrasplenic steady-state dendritic cell precursors that are distinct from monocytes. *Nat. Immunol.* **7,** 663–671 (2006).
187. Roche, P. A. & Furuta, K. The ins and outs of MHC class II-mediated antigen processing and presentation. *Nat. Rev. Immunol.* **15,** 203–216 (2015).
188. Cybulsky, M. I., Cheong, C. & Robbins, C. S. Macrophages and Dendritic Cells: Partners in Atherogenesis. *Circ. Res.* **118,** 637–652 (2016).
189. van Kasteren, S. I., Overkleeft, H., Ovaa, H. & Neefjes, J. Chemical biology of antigen presentation by MHC molecules. *Curr. Opin. Immunol.* **26,** 21–31 (2014).
190. Sage, A. P. *et al.* MHC Class II-restricted antigen presentation by plasmacytoid dendritic cells drives proatherogenic T cell immunity. *Circulation* **130,** 1363–1373 (2014).
191. Vega-Ramos, J., Roquilly, A., Asehnoune, K. & Villadangos, J. A. Modulation of dendritic cell antigen presentation by pathogens, tissue damage and secondary inflammatory signals. *Curr. Opin. Pharmacol.* **17,** 64–70 (2014).
192. Rossjohn, J. *et al.* T cell antigen receptor recognition of antigen-presenting molecules. *Annu. Rev. Immunol.* **33,** 169–200 (2015).
193. van de Weijer, M. L., Luteijn, R. D. & Wiertz, E. J. H. J. Viral immune evasion: Lessons in MHC class I antigen presentation. *Semin. Immunol.* **27,** 125–137 (2015).
194. Gerdes, N. & Zirlik, A. Co-stimulatory molecules in and beyond co-stimulation - tipping the balance in atherosclerosis? *Thromb. Haemost.* **106,** 804–813 (2011).
195. Gotsman, I., Sharpe, A. H. & Lichtman, A. H. T-cell costimulation and coinhibition in atherosclerosis. *Circulation Research* **103,** 1220–1231 (2008).

196. Curtsinger, J. M. & Mescher, M. F. Inflammatory cytokines as a third signal for T cell activation. *Curr. Opin. Immunol.* **22,** 333–340 (2010).

197. Fatkhullina, A. R., Peshkova, I. O. & Koltsova, E. K. The Role of Cytokines in the Development of Atherosclerosis. *Biochemistry. (Mosc).* **81,** 1358–1370 (2016).

198. Ramji, D. P. & Davies, T. S. Cytokines in atherosclerosis: Key players in all stages of disease and promising therapeutic targets. *Cytokine Growth Factor Rev.* **26,** 673–685 (2015).

199. Moudgil, K. D. Interplay among cytokines and T cell subsets in the progression and control of immune-mediated diseases. *Cytokine* **74,** 1–4 (2015).

200. Worbs, T., Hammerschmidt, S. I. & Forster, R. Dendritic cell migration in health and disease. *Nat. Rev. Immunol.* **17,** 30–48 (2017).

201. Libby, P., Lichtman, A. & Hansson, G. Immune effector mechanisms implicated in atherosclerosis: from mice to humans. *Immunity* **38,** 1092–1104 (2013).

202. Stemme, S. *et al.* T lymphocytes from human atherosclerotic plaques recognize oxidized low density lipoprotein. *Proc. Natl. Acad. Sci. U. S. A.* **92,** 3893–3897 (1995).

203. Pierides, C., Bermudez-Fajardo, A., Fredrikson, G. N., Nilsson, J. & Oviedo-Orta, E. Immune responses elicited by apoB-100-derived peptides in mice. *Immunol. Res.* **56,** 96–108 (2013).

204. Wick, G., Jakic, B., Buszko, M., Wick, M. C. & Grundtman, C. The role of heat shock proteins in atherosclerosis. *Nat. Rev. Cardiol.* **11,** 516–529 (2014).

205. Kimura, T., Tse, K., Sette, A. & Ley, K. Vaccination to modulate atherosclerosis. *Autoimmunity* **48,** 152–160 (2015).

206. Kimura, T. *et al.* Atheroprotective vaccination with MHC-II-restricted ApoB peptides induces peritoneal IL-10-producing CD4 T cells. *Am. J. Physiol. Heart Circ. Physiol.* **312,** H781–H790 (2017).

207. Butcher, E. C. & Picker, L. J. Lymphocyte homing and homeostasis. *Science* **272,** 60–66 (1996).

208. Fearon, D. T. The expansion and maintenance of antigen-selected CD8(+) T cell clones. *Adv. Immunol.* **96,** 103–139 (2007).

209. van Panhuys, N. TCR Signal Strength Alters T-DC Activation and Interaction Times and Directs the Outcome of Differentiation. *Front. Immunol.* **7,** 6 (2016).

210. Zhou, X., Nicoletti, A., Elhage, R. & Hansson, G. K. Transfer of CD4(+) T cells aggravates atherosclerosis in immunodeficient apolipoprotein E knockout mice. *Circulation* **102,** 2919–2922 (2000).

211. Gewaltig, J., Kummer, M., Koella, C., Cathomas, G. & Biedermann, B. C. Requirements for CD8 T-cell migration into the human arterial wall. *Hum. Pathol.* **39,** 1756–1762 (2008).

212. Olson, N. C. *et al.* Decreased naive and increased memory CD4(+) T cells are associated with subclinical atherosclerosis: the multi-ethnic study of atherosclerosis. *PLoS One* **8,** e71498 (2013).

213. Behnamfar, N., Zibaeenezhad, M. J., Golmoghaddam, H. & Doroudchi, M. CD45RO+ memory T-cells produce IL-17 in patients with atherosclerosis. *Cell. Mol. Biol. (Noisy-le-grand).* **61,** 17–23 (2015).

214. Jonasson, L., Holm, J., Skalli, O., Bondjers, G. & Hansson, G. K. Regional accumulations of T cells, macrophages, and smooth muscle cells in the human atherosclerotic plaque. *Arteriosclerosis* **6,** 131–138 (1986).

215. Norment, A. M., Salter, R. D., Parham, P., Engelhard, V. H. & Littman, D. R. Cell-cell adhesion mediated by CD8 and MHC class I molecules. *Nature* **336,** 79–81 (1988).

216. Doyle, C. & Strominger, J. L. Interaction between CD4 and class II MHC molecules mediates cell adhesion. *Nature* **330,** 256–259 (1987).

217. Wieczorek, M. *et al.* Major Histocompatibility Complex (MHC) Class I and MHC Class

II Proteins: Conformational Plasticity in Antigen Presentation. *Front. Immunol.* **8,** 292 (2017).

218. van Dijk, R. A. *et al.* A change in inflammatory footprint precedes plaque instability: a systematic evaluation of cellular aspects of the adaptive immune response in human atherosclerosis. *J. Am. Heart Assoc.* **4,** (2015).
219. Cochain, C. *et al.* CD8+ T Cells Regulate Monopoiesis and Circulating Ly6C-high Monocyte Levels in Atherosclerosis in Mice. *Circ. Res.* **117,** 244–253 (2015).
220. Kyaw, T. *et al.* Cytotoxic and proinflammatory CD8+ T lymphocytes promote development of vulnerable atherosclerotic plaques in apoE-deficient mice. *Circulation* **127,** 1028–1039 (2013).
221. Cochain, C. & Zernecke, A. Protective and pathogenic roles of CD8+ T cells in atherosclerosis. *Basic Res. Cardiol.* **111,** 71 (2016).
222. Clement, M. *et al.* Control of the T follicular helper-germinal center B-cell axis by CD8(+) regulatory T cells limits atherosclerosis and tertiary lymphoid organ development. *Circulation* **131,** 560–570 (2015).
223. Chyu, K.-Y. *et al.* CD8+ T cells mediate the athero-protective effect of immunization with an ApoB-100 peptide. *PLoS One* **7,** e30780 (2012).
224. Hirahara, K. & Nakayama, T. CD4+ T-cell subsets in inflammatory diseases: beyond the Th1/Th2 paradigm. *Int. Immunol.* **28,** 163–171 (2016).
225. Tse, K., Tse, H., Sidney, J., Sette, A. & Ley, K. T cells in atherosclerosis. *Int. Immunol.* **25,** 615–622 (2013).
226. Buono, C. *et al.* T-bet deficiency reduces atherosclerosis and alters plaque antigen-specific immune responses. *Proc. Natl. Acad. Sci. U. S. A.* **102,** 1596–1601 (2005).
227. Whitman, S. C., Ravisankar, P., Elam, H. & Daugherty, A. Exogenous interferon-gamma enhances atherosclerosis in apolipoprotein E-/- mice. *Am. J. Pathol.* **157,** 1819–1824 (2000).
228. Zheng, W. & Flavell, R. A. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell* **89,** 587–596 (1997).
229. Wurtz, O., Bajenoff, M. & Guerder, S. IL-4-mediated inhibition of IFN-gamma production by CD4+ T cells proceeds by several developmentally regulated mechanisms. *Int. Immunol.* **16,** 501–508 (2004).
230. Cornicelli, J. A., Butteiger, D., Rateri, D. L., Welch, K. & Daugherty, A. Interleukin-4 augments acetylated LDL-induced cholesterol esterification in macrophages. *J. Lipid Res.* **41,** 376–383 (2000).
231. King, V. L., Szilvassy, S. J. & Daugherty, A. Interleukin-4 deficiency decreases atherosclerotic lesion formation in a site-specific manner in female LDL receptor-/- mice. *Arterioscler. Thromb. Vasc. Biol.* **22,** 456–461 (2002).
232. Binder, C. J. *et al.* IL-5 links adaptive and natural immunity specific for epitopes of oxidized LDL and protects from atherosclerosis. *J. Clin. Invest.* **114,** 427–437 (2004).
233. Laurence, A. & O'Shea, J. J. T(H)-17 differentiation: of mice and men. *Nature immunology* **8,** 903–905 (2007).
234. Erbel, C. *et al.* Inhibition of IL-17A attenuates atherosclerotic lesion development in apoE-deficient mice. *J. Immunol.* **183,** 8167–8175 (2009).
235. Cheng, X. *et al.* Inhibition of IL-17A in atherosclerosis. *Atherosclerosis* **215,** 471–474 (2011).
236. Su, Z. *et al.* IFN-gamma-producing Th17 cells bias by HMGB1-T-bet/RUNX3 axis might contribute to progression of coronary artery atherosclerosis. *Atherosclerosis* **243,** 421–428 (2015).
237. Tiniakou, I. *et al.* High-density lipoprotein attenuates Th1 and th17 autoimmune

responses by modulating dendritic cell maturation and function. *J. Immunol.* **194,** 4676–4687 (2015).

238. Lee, Y. K., Mukasa, R., Hatton, R. D. & Weaver, C. T. Developmental plasticity of Th17 and Treg cells. *Curr. Opin. Immunol.* **21,** 274–280 (2009).
239. Spitz, C. *et al.* Regulatory T cells in atherosclerosis: critical immune regulatory function and therapeutic potential. *Cell. Mol. Life Sci.* **73,** 901–922 (2016).
240. Chaudhry, A. *et al.* Interleukin-10 signaling in regulatory T cells is required for suppression of Th17 cell-mediated inflammation. *Immunity* **34,** 566–578 (2011).
241. Wang, Z. *et al.* Effect of hyperlipidemia on Foxp3 expression in apolipoprotein E-knockout mice. *J. Cardiovasc. Med. (Hagerstown).* **15,** 273–279 (2014).
242. Wigren, M. *et al.* Low levels of circulating CD4+FoxP3+ T cells are associated with an increased risk for development of myocardial infarction but not for stroke. *Arterioscler. Thromb. Vasc. Biol.* **32,** 2000–2004 (2012).
243. Liu, G. *et al.* Phenotypic and functional switch of macrophages induced by regulatory CD4+CD25+ T cells in mice. *Immunol. Cell Biol.* **89,** 130–142 (2011).
244. Mallat, Z. *et al.* Induction of a regulatory T cell type 1 response reduces the development of atherosclerosis in apolipoprotein E-knockout mice. *Circulation* **108,** 1232–1237 (2003).
245. Foks, A. C. *et al.* Differential effects of regulatory T cells on the initiation and regression of atherosclerosis. *Atherosclerosis* **218,** 53–60 (2011).
246. Foks, A. C., Lichtman, A. H. & Kuiper, J. Treating atherosclerosis with regulatory T cells. *Arterioscler. Thromb. Vasc. Biol.* **35,** 280–287 (2015).
247. Lin, Y. *et al.* Circulating Th22 and Th9 levels in patients with acute coronary syndrome. *Mediators Inflamm.* **2013,** 635672 (2013).
248. Gong, F. *et al.* The paradoxical role of IL-17 in atherosclerosis. *Cell. Immunol.* **297,** 33–39 (2015).
249. Liu, A. Y. *et al.* Mast cells recruited to mesenteric lymph nodes during helminth infection remain hypogranular and produce IL-4 and IL-6. *J. Immunol.* **190,** 1758–1766 (2013).
250. Grivel, J.-C. *et al.* Activation of T lymphocytes in atherosclerotic plaques. *Arterioscler. Thromb. Vasc. Biol.* **31,** 2929–2937 (2011).
251. Lee, H. J. *et al.* GATA-3 induces T helper cell type 2 (Th2) cytokine expression and chromatin remodeling in committed Th1 cells. *J. Exp. Med.* **192,** 105–115 (2000).
252. Butcher, M. J. *et al.* Atherosclerosis-Driven Treg Plasticity Results in Formation of a Dysfunctional Subset of Plastic IFNgamma+ Th1/Tregs. *Circ. Res.* **119,** 1190–1203 (2016).
253. Das, R., Sant'Angelo, D. B. & Nichols, K. E. Transcriptional control of invariant NKT cell development. *Immunol. Rev.* **238,** 195–215 (2010).
254. Godfrey, D. I. & Kronenberg, M. Going both ways: immune regulation via CD1d-dependent NKT cells. *J. Clin. Invest.* **114,** 1379–1388 (2004).
255. Skold, M., Faizunnessa, N. N., Wang, C. R. & Cardell, S. CD1d-specific NK1.1+ T cells with a transgenic variant TCR. *J. Immunol.* **165,** 168–174 (2000).
256. Thomas, S. Y. *et al.* CD1d-restricted NKT cells express a chemokine receptor profile indicative of Th1-type inflammatory homing cells. *J. Immunol.* **171,** 2571–2580 (2003).
257. Qiao, X. *et al.* OCH-mediated shift of Th1 and Th2 cytokines by NKT cells in mice with aplastic anemia. *Med. Oncol.* **32,** 67 (2015).
258. Bondarenko, S., Catapano, A. L. & Norata, G. D. The CD1d-natural killer T cell axis in atherosclerosis. *J. Innate Immun.* **6,** 3–12 (2014).
259. Aslanian, A. M., Chapman, H. A. & Charo, I. F. Transient role for CD1d-restricted natural killer T cells in the formation of atherosclerotic lesions. *Arterioscler. Thromb. Vasc. Biol.*

25, 628–632 (2005).

260. van Puijvelde, G. H. M. & Kuiper, J. NKT cells in cardiovascular diseases. *Eur. J. Pharmacol.* (2017). doi:10.1016/j.ejphar.2017.03.052

261. Rogers, L. *et al.* Deficiency of invariant V alpha 14 natural killer T cells decreases atherosclerosis in LDL receptor null mice. *Cardiovasc. Res.* **78,** 167–174 (2008).

262. Li, Y. *et al.* CD4+ natural killer T cells potently augment aortic root atherosclerosis by perforin- and granzyme B-dependent cytotoxicity. *Circ. Res.* **116,** 245–254 (2015).

263. Nakai, Y. *et al.* Natural killer T cells accelerate atherogenesis in mice. *Blood* **104,** 2051–2059 (2004).

264. Wang, S. *et al.* IRE1alpha-XBP1s induces PDI expression to increase MTP activity for hepatic VLDL assembly and lipid homeostasis. *Cell Metab.* **16,** 473–486 (2012).

265. Brozovic, S. *et al.* CD1d function is regulated by microsomal triglyceride transfer protein. *Nat. Med.* **10,** 535–539 (2004).

266. VanderLaan, P. A. *et al.* Characterization of the natural killer T-cell response in an adoptive transfer model of atherosclerosis. *Am. J. Pathol.* **170,** 1100–1107 (2007).

267. Tsiantoulas, D., Diehl, C. J., Witztum, J. L. & Binder, C. J. B cells and humoral immunity in atherosclerosis. *Circ. Res.* **114,** 1743–1756 (2014).

268. Hilgendorf, I. *et al.* Innate response activator B cells aggravate atherosclerosis by stimulating T helper-1 adaptive immunity. *Circulation* **129,** 1677–1687 (2014).

269. Rosenfeld, S. M. *et al.* B-1b Cells Secrete Atheroprotective IgM and Attenuate Atherosclerosis. *Circ. Res.* **117,** e28-39 (2015).

270. Major, A. S., Fazio, S. & Linton, M. F. B-lymphocyte deficiency increases atherosclerosis in LDL receptor-null mice. *Arterioscler. Thromb. Vasc. Biol.* **22,** 1892–1898 (2002).

271. Kyaw, T., Tipping, P., Bobik, A. & Toh, B.-H. Opposing roles of B lymphocyte subsets in atherosclerosis. *Autoimmunity* **50,** 52–56 (2017).

272. Kyaw, T. *et al.* B1a B lymphocytes are atheroprotective by secreting natural IgM that increases IgM deposits and reduces necrotic cores in atherosclerotic lesions. *Circ. Res.* **109,** 830–840 (2011).

273. Kyaw, T. *et al.* Depletion of B2 but not B1a B cells in BAFF receptor-deficient ApoE mice attenuates atherosclerosis by potently ameliorating arterial inflammation. *PLoS One* **7,** e29371 (2012).

274. Lewis, M. J. *et al.* Immunoglobulin M is required for protection against atherosclerosis in low-density lipoprotein receptor-deficient mice. *Circulation* **120,** 417–426 (2009).

275. Hermansson, A. *et al.* Inhibition of T cell response to native low-density lipoprotein reduces atherosclerosis. *J. Exp. Med.* **207,** 1081–1093 (2010).

276. Tsiantoulas, D. *et al.* Increased Plasma IgE Accelerate Atherosclerosis in Secreted IgM Deficiency. *Circ. Res.* **120,** 78–84 (2017).

277. Lu, H. & Daugherty, A. Regulatory B cells, interleukin-10, and atherosclerosis. *Current opinion in lipidology* **26,** 470–471 (2015).

278. Strom, A. C. *et al.* B regulatory cells are increased in hypercholesterolaemic mice and protect from lesion development via IL-10. *Thromb. Haemost.* **114,** 835–847 (2015).

279. Sage, A. P. *et al.* Regulatory B cell-specific interleukin-10 is dispensable for atherosclerosis development in mice. *Arterioscler. Thromb. Vasc. Biol.* **35,** 1770–1773 (2015).

280. Grasset, E. K. *et al.* Sterile inflammation in the spleen during atherosclerosis provides oxidation-specific epitopes that induce a protective B-cell response. *Proc. Natl. Acad. Sci. U. S. A.* **112,** E2030-8 (2015).

281. Hamaguchi, Y. *et al.* The peritoneal cavity provides a protective niche for B1 and conventional B lymphocytes during anti-CD20 immunotherapy in mice. *J. Immunol.* **174,** 4389–4399 (2005).

282. Ma, H. & Kovanen, P. T. IgE-dependent generation of foam cells: an immune mechanism involving degranulation of sensitized mast cells with resultant uptake of LDL by macrophages. *Arterioscler. Thromb. Vasc. Biol.* **15,** 811–819 (1995).

283. Pende, A., Artom, N., Bertolotto, M., Montecucco, F. & Dallegri, F. Role of neutrophils in atherogenesis: an update. *Eur. J. Clin. Invest.* **46,** 252–263 (2016).

284. Martin, C. *et al.* Chemokines acting via CXCR2 and CXCR4 control the release of neutrophils from the bone marrow and their return following senescence. *Immunity* **19,** 583–593 (2003).

285. Suratt, B. T. *et al.* Neutrophil maturation and activation determine anatomic site of clearance from circulation. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **281,** L913-21 (2001).

286. Kolaczkowska, E. & Kubes, P. Neutrophil recruitment and function in health and inflammation. *Nat. Rev. Immunol.* **13,** 159–175 (2013).

287. Hartl, D. *et al.* Infiltrated Neutrophils Acquire Novel Chemokine Receptor Expression and Chemokine Responsiveness in Chronic Inflammatory Lung Diseases. *J. Immunol.* **181,** 8053–8067 (2008).

288. Drechsler, M., Megens, R. T. A., van Zandvoort, M., Weber, C. & Soehnlein, O. Hyperlipidemia-triggered neutrophilia promotes early atherosclerosis. *Circulation* **122,** 1837–1845 (2010).

289. Sundd, P. *et al.* 'Slings' enable neutrophil rolling at high shear. *Nature* **488,** 399–403 (2012).

290. Lingrel, J. B. *et al.* Myeloid-specific Kruppel-like factor 2 inactivation increases macrophage and neutrophil adhesion and promotes atherosclerosis. *Circ. Res.* **110,** 1294–1302 (2012).

291. Doring, Y., Drechsler, M., Soehnlein, O. & Weber, C. Neutrophils in atherosclerosis: from mice to man. *Arterioscler. Thromb. Vasc. Biol.* **35,** 288–295 (2015).

292. Soehnlein, O., Zernecke, A. & Weber, C. Neutrophils launch monocyte extravasation by release of granule proteins. *Thromb. Haemost.* **102,** 198–205 (2009).

293. Tiyerili, V. *et al.* Neutrophil-derived myeloperoxidase promotes atherogenesis and neointima formation in mice. *Int. J. Cardiol.* **204,** 29–36 (2016).

294. Maeda, K., Yasunari, K., Sato, E. F. & Inoue, M. Enhanced oxidative stress in neutrophils from hyperlipidemic guinea pig. *Atherosclerosis* **181,** 87–92 (2005).

295. Leclercq, A. *et al.* Involvement of intraplaque hemorrhage in atherothrombosis evolution via neutrophil protease enrichment. *J. Leukoc. Biol.* **82,** 1420–1429 (2007).

296. Naruko, T. *et al.* Neutrophil infiltration of culprit lesions in acute coronary syndromes. *Circulation* **106,** 2894–2900 (2002).

297. Bot, I. *et al.* CXCR4 blockade induces atherosclerosis by affecting neutrophil function. *J. Mol. Cell. Cardiol.* **74,** 44–52 (2014).

298. Branzk, N. *et al.* Neutrophils sense microbe size and selectively release neutrophil extracellular traps in response to large pathogens. *Nat. Immunol.* **15,** 1017–1025 (2014).

299. Megens, R. T. A. *et al.* Presence of luminal neutrophil extracellular traps in atherosclerosis. *Thrombosis and haemostasis* **107,** 597–598 (2012).

300. Doring, Y., Soehnlein, O. & Weber, C. Neutrophil Extracellular Traps in Atherosclerosis and Atherothrombosis. *Circ. Res.* **120,** 736–743 (2017).

301. Warnatsch, A., Ioannou, M., Wang, Q. & Papayannopoulos, V. Inflammation. Neutrophil extracellular traps license macrophages for cytokine production in atherosclerosis. *Science* **349,** 316–320 (2015).

302. Wezel, A. *et al.* Mast cells mediate neutrophil recruitment during atherosclerotic plaque progression. *Atherosclerosis* **241,** 289–296 (2015).

303. Bot, I., Shi, G.-P. & Kovanen, P. T. Mast cells as effectors in atherosclerosis. *Arterioscler.*

Thromb. Vasc. Biol. **35,** 265–271 (2015).

304. Kaartinen, M., Penttila, A. & Kovanen, P. T. Mast cells of two types differing in neutral protease composition in the human aortic intima. Demonstration of tryptase- and tryptase/chymase-containing mast cells in normal intimas, fatty streaks, and the shoulder region of atheromas. *Arterioscler. Thromb. a J. Vasc. Biol.* **14,** 966–972 (1994).
305. Kovanen, P. T., Kaartinen, M. & Paavonen, T. Infiltrates of activated mast cells at the site of coronary atheromatous erosion or rupture in myocardial infarction. *Circulation* **92,** 1084–1088 (1995).
306. Bot, I. *et al.* Perivascular mast cells promote atherogenesis and induce plaque destabilization in apolipoprotein E-deficient mice. *Circulation* **115,** 2516–2525 (2007).
307. El-Agamy, D. S. Targeting c-kit in the therapy of mast cell disorders: current update. *European journal of pharmacology* **690,** 1–3 (2012).
308. Wernersson, S. & Pejler, G. Mast cell secretory granules: armed for battle. *Nat. Rev. Immunol.* **14,** 478–494 (2014).
309. Dahlin, J. S. & Hallgren, J. Mast cell progenitors: origin, development and migration to tissues. *Mol. Immunol.* **63,** 9–17 (2015).
310. Galli, S. J., Borregaard, N. & Wynn, T. a. Phenotypic and functional plasticity of cells of innate immunity: macrophages, mast cells and neutrophils. *Nat. Immunol.* **12,** 1035–44 (2011).
311. Kraft, S. & Kinet, J.-P. New developments in FcepsilonRI regulation, function and inhibition. *Nature reviews. Immunology* **7,** 365–378 (2007).
312. Varadaradjalou, S. *et al.* Toll-like receptor 2 (TLR2) and TLR4 differentially activate human mast cells. **2,** 899–906 (2003).
313. Erdei, A., Andrasfalvy, M., Peterfy, H., Toth, G. & Pecht, I. Regulation of mast cell activation by complement-derived peptides. *Immunol. Lett.* **92,** 39–42 (2004).
314. Bot, I. *et al.* The neuropeptide substance P mediates adventitial mast cell activation and induces intraplaque hemorrhage in advanced atherosclerosis. *Circ. Res.* **106,** 89–92 (2010).
315. Bot, I. *et al.* Mast cell chymase inhibition reduces atherosclerotic plaque progression and improves plaque stability in ApoE-/- mice. *Cardiovasc. Res.* **89,** 244–252 (2011).
316. Johnson, J. L., Jackson, C. L., Angelini, G. D. & George, S. J. Activation of matrix-degrading metalloproteinases by mast cell proteases in atherosclerotic plaques. *Arterioscler. Thromb. Vasc. Biol.* **18,** 1707–1715 (1998).
317. Lee, M., Calabresi, L., Chiesa, G., Franceschini, G. & Kovanen, P. T. Mast cell chymase degrades apoE and apoA-II in apoA-I-knockout mouse plasma and reduces its ability to promote cellular cholesterol efflux. *Arterioscler. Thromb. Vasc. Biol.* **22,** 1475–1481 (2002).
318. Zhang, J. *et al.* Chemokine (C-C motif) receptor 2 mediates mast cell migration to abdominal aortic aneurysm lesions in mice. *Cardiovasc. Res.* **96,** 543–51 (2012).
319. Kokkonen, J. O. & Kovanen, P. T. Low density lipoprotein degradation by rat mast cells. Demonstration of extracellular proteolysis caused by mast cell granules. *J. Biol. Chem.* **260,** 14756–14763 (1985).
320. Tanaka, M. *et al.* Eosinophil count is positively correlated with coronary artery calcification. *Hypertens. Res.* **35,** 325–328 (2012).
321. Selathurai, A. *et al.* Natural killer (NK) cells augment atherosclerosis by cytotoxic-dependent mechanisms. *Cardiovasc. Res.* **102,** 128–137 (2014).
322. Engelbertsen, D. *et al.* Expansion of CD25+ Innate Lymphoid Cells Reduces Atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* **35,** 2526–2535 (2015).
323. Foks, A. C. *et al.* CD11b+Gr-1+ myeloid-derived suppressor cells reduce atherosclerotic

lesion development in LDLr deficient mice. *Cardiovasc. Res.* **111,** 252–261 (2016).

324. Vu, D. M. *et al.* gammadeltaT cells are prevalent in the proximal aorta and drive nascent atherosclerotic lesion progression and neutrophilia in hypercholesterolemic mice. *PLoS One* **9,** e109416 (2014).
325. Matsuura, E. *et al.* Is atherosclerosis an autoimmune disease? *BMC medicine* **12,** 47 (2014).
326. Siess, W. Platelet interaction with bioactive lipids formed by mild oxidation of low-density lipoprotein. *Pathophysiol. Haemost. Thromb.* **35,** 292–304 (2006).
327. Wang, L., Knudsen, E., Jin, Y., Gessani, S. & Maghazachi, A. A. Lysophospholipids and chemokines activate distinct signal transduction pathways in T helper 1 and T helper 2 cells. *Cell. Signal.* **16,** 991–1000 (2004).
328. Bot, M. *et al.* Lysophosphatidic acid triggers mast cell-driven atherosclerotic plaque destabilization by increasing vascular inflammation. *J. Lipid Res.* **54,** 1265–74 (2013).
329. Chen, L. *et al.* Lysophosphatidic acid directly induces macrophage-derived foam cell formation by blocking the expression of SRBI. *Biochem. Biophys. Res. Commun.* (2017). doi:10.1016/j.bbrc.2017.07.159

Chapter 2

The impact of mast cells on cardiovascular diseases

Eur J Pharmacol. 2016; 778:103-15

[1]Eva Kritikou
[1]Johan Kuiper
[2]Petri T. Kovanen
[1]Ilze Bot

[1] Division of Biopharmaceutics, Leiden Academic Centre for Drug Research, Leiden University, Leiden, The Netherlands

[2]Wihuri Research Institute, Helsinki, Finland

Abstract

Mast cells comprise an innate immune cell population, which accumulates in tissues proximal to the outside environment and, upon activation, augments the progression of immunological reactions through the release and diffusion of either pre-formed or newly generated mediators. The released products of mast cells include histamine, proteases, as well as a variety of cytokines, chemokines and growth factors, which act on the surrounding microenvironment thereby shaping the immune responses triggered in various diseased states. Mast cells have also been detected in the vessel wall and are implicated in the onset and progression of numerous cardiovascular diseases. Notably, modulation of distinct mast cell actions using genetic and pharmacological approaches highlights the crucial role of this cell type in cardiovascular syndromes. The acquired evidence renders mast cells and their mediators potential prognostic markers and therapeutic targets in a broad spectrum of pathophysiological conditions related to cardiovascular diseases.

1. Introduction

Mast cells are innate immune cells characterized by a monolobular nucleus and numerous cytoplasmic granules[1], morphological features which distinguish them from a variety of cell types that comprise the immune system. Originating from hematopoietic stem cells within the bone marrow[2], mast cell progenitors are released into the circulation and, upon the influence of cytokine and chemokine signals, home in tissues and further differentiate into mature mast cells[3]. The place of maturation for mast cells is either mucosal surfaces or connective tissue, therefore granting them a wide distribution throughout the body. Tissue resident mature mast cells exert their effector functions after activation triggered by cytokines, antibodies and proteins specific for receptors present on their surface. The most widely studied mast cell activation pathway is the antigen-specific activation by Immunoglobulin-E (IgE) antibodies, which bind to the high affinity membrane receptor Fcepsilon Receptor-1 (FcεR1)[4]. However, during the last decades, additional agents triggering mast cell activation have been discovered, such as the Immunoglobulin-G (IgG) antibodies[5], complement system components[6], but also pathogenic Toll-like receptor peptides[7], as well as endogenous ligands like substance P (SP)[8] and endothelin-1 (ET-1)[9].

The mast cell secretory granules contain a variety of molecules such as proteoglycans (e.g. heparin and chondroitin sulfate), histamine, cysteinyl cathepsins, proteases (e.g. chymase and tryptase) and a broad spectrum of pro-inflammatory as well as anti-inflammatory cytokines[10]. Through their mediator release, mast cells are able to act on the adjacent cells and shape the local microenvironment.

In addition, mast cells residing in different tissues exhibit a diverse protease content, and can consequently be separated into various subpopulations. However, despite the differences in the granule protease composition of each mature mast cell subtype, their content can be actively shaped depending on signals received from the surrounding microenvironment[11,12]. Finally, the ultimate protease phenotype in a tissue may switch from one to another in a reversible way. Such *de novo* formation of mast cell mediators, which extends itself beyond the granule proteases, depends not only on the intrinsic characteristics of each tissue but also on local pathologic conditions at any given point, granting remarkable plasticity to mast cells regarding their effector functions[13,14].

Within the cardiovascular system, mast cells reside in close proximity to blood vessels, as well as in the heart of both humans and rodents[15], where they participate in physiological functions such as angiogenesis and local generation of the vasoconstrictive hormone Angiotensin II (Ang II)[16]. The majority of mast cells populating the human heart and vessels consist mainly of tryptase[17], while in mice they are recognized as connectice

tissue mast cells and contain granules filled with heparin, chymase and tryptase[18,19]. However, particularly in humans, the mast cell protease content in the vessels shows remarkable variation among subjects[20], attributing each protease a differential role in the pathophysiology of cardiovascular diseases. Yet, aside from proteases, there is a plethora of additional mast cell mediators which participate in the pathological events observed in cardiovascular syndromes (**Figure 1**).

Mast cells in the vasculature are mainly distinguished for their adverse effects in syndromes such as abdominal aortic aneurysm[21], myocardial infarction[22], and atherosclerosis[23], displaying thus a crucial role in the leading cause of death worldwide[24]. It is therefore intriguing to pinpoint the overall importance of mast cells in a wide variety of such conditions.

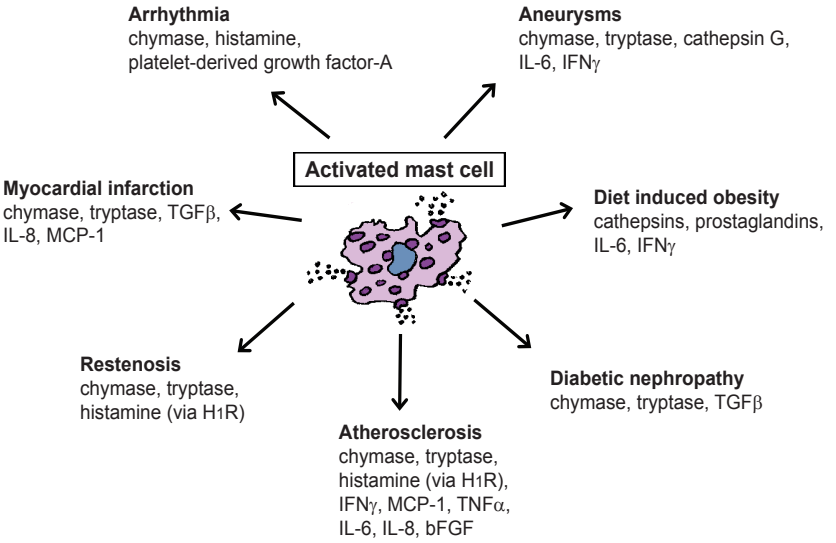


Figure 1: Mast cells in cardiovascular diseases. This figure depicts major cardiovascular diseases in which mast cells have been implicated. Specific mast cell mediators, summarized here, have been reported in each of these syndromes. *Abbreviations: IL-6: Interleukin-6, IL-8: Interleukin-8, TNFα: tumor necrosis factor-alpha, IFNγ: Interferon-gamma, TGF-β: transforming growth factor-beta, MCP-1: monocyte chemoattractant protein-1, bFGF: basic fibroblast growth factor.*

2. Diet-induced obesity

The incidence of obesity, due to high fat diet, has become a Western society epidemic and is closely linked to type 2 diabetes, as well as other metabolic and cardiac disorders[25]. Increased chronic inflammation at a low degree is observed in white adipose tissue (WAT) of obese humans and mice, with local infiltration of macrophages[26] and T cells[27]. Mast cells have also been detected in obese adipose tissue, located next

to microvessels and are directly associated with the pathology of this disorder. More specifically, Liu et al. reported in 2009 an increased number of mast cells in WAT of obese humans and mice, accompanied by elevated serum tryptase levels as well as local and systemic levels of inflammatory cytokines, chemokines and proteases, compared to lean subjects. Here, mast cells contribute to WAT apoptosis and angiogenesis. This effect is exerted *via* Interleukin-6 (IL-6) and Interferon-gamma (IFNγ) cytokines, which in turn increase cysteinyl cathepsin expression, thus promoting diet-induced obesity. Importantly, in the cited study, mast cells are observed to infiltrate obese WAT prior to macrophages. Likewise, mast cells are shown to co-localize with $CD8^+$ T cells in mouse WAT[28], suggesting a role in adipose tissue inflammation. An additional study reported that mast cell deficient, $Kit^{W\text{-}sh/W\text{-}sh}$, mice transplanted with hematopoietic-prostaglandin synthase deficient mast cells are not able to gain weight similarly as mice transplanted with wild-type mast cells, pinpointing the importance of mast cell derived prostaglandins in adipose tissue function[29]. As adipocytes themselves are also an important source of cytokines[30], this adds up to the local inflammatory burden. Interestingly, adipocytokines have been linked to mast cells in the context of allergic inflammation and asthma[31], introducing a possible crosstalk between the resident cells of adipose tissue and the infiltrating mast cells.

2.1. Type II-diabetes mellitus

Notably, obesity comprises an essential risk factor for the development of type 2-diabetes mellitus[32]. Non-insulin dependent diabetes is a metabolic disease defined by hyperglycemia and insulin-resistance, which is greatly influenced by obesity. Aside from their role in obesity, mast cells have been directly linked to type 2-diabetes. Mice fed a high-fat diet while lacking mast cells show higher glucose tolerance, compared to the wild type strain[33]. Since tumor necrosis factor-alpha (TNFα) was found to be overexpressed in obese mice[34], this cytokine has been considered a key mediator in the induction of insulin resistance[35]. However in experiments using TNFα deficient mast cells[33], TNFα did not contribute to the effect of mast cells in obesity, indicating that the metabolic changes induced by this cytokine may have been due to TNFα derived from other inflammatory cells than mast cells.

2.2. Diabetic nephropathy

Diabetes mellitus can often lead to serious renal complications, such as diabetic nephropathy, a condition showing increasing mortality rates over the years[36]. Diabetic nephropathy is marked by glomerular and tubular basement membrane thickening and hypertrophic mesangial matrix, while it can lead to tubulointerstitial fibrosis and

glomerulosclerosis[37]. In the past, human mast cells have been reported to infiltrate the diseased kidney at increasing numbers as diabetic nephropathy progresses[38], while participating in the initial mechanisms of tubulointerstitial injury[39]. Furthermore, mast cell infiltration in the kidneys of rodents was clearly established in renal diseases, the mast cell mediators chymase, tryptase and transforming growth factor-beta$_1$ (TGF-β_1) showing increased levels in injured rat kidney tissue[40]. Importantly, TGF-β_1 has already been reported to be a key cytokine responsible for the aggravation of renal fibrosis[41], *via* collagen overexpression by fibroblasts[42]. Chymase has been connected to various mechanisms that shape renal diseases[43], and regarding diabetes and diabetic nephropathy, chymase was suggested to participate in local Ang II formation[44]. In addition, tryptase was reported to enhance renal fibrosis while additionally being implicated in collagen synthesis, which illustrates a potential regulatory mechanism of mast cells on fibroblasts[45]. In fact, diabetic rats treated with the mast cell stabilizer tranilast displayed diminished chymase-positive mast cell infiltration in mesenteric vessels, diminished mesenteric vascular collagen deposition, and ultimately amelioration of the diabetes-induced vessel fibrosis[46], which was considered to result from inhibition of chymase and subsequent reduction in the generation of Ang II.

Seemingly, mast cells in both obesity and diabetes function either by establishing cell interactions with local and infiltrating cell types, or *via* their degranulation products acting *in situ*. Through these routes, mast cells may contribute to various life-threatening cardiovascular syndromes. For instance, in an experimental diabetes model, mice showed increased cardiac mast cell activation and defective heart function, which led to the development of cardiomyopathy[47]. However, upon treatment with the mast cell stabilizer nedocromyl, the cardiac mast cell numbers were decreased, followed by decreased collagen deposition and normalized cytokine levels. Furthermore, in a recent patient study examining obese subjects for subclinical atherosclerosis, serum tryptase positively correlated with metabolic risk factors, such as body-mass index[48], as well as atherosclerosis markers, suggesting that mast cell activation composes a tight link between obesity and cardiometabolic diseases.

3. Atherosclerosis

Atherosclerosis is the primary underlying cause of acute cardiovascular syndromes, such as stroke and myocardial infarction, principal causes of death in Western society[49]. This vascular disease is a chronic inflammatory condition, characterized by thickening of the arterial wall after lipid accumulation, which results in the formation of an atheromatous plaque[50]. Elevated levels of cholesterol-carrying low density lipoprotein (LDL), and its subsequent penetration through the vascular endothelial cell layer where it gets oxidized (oxLDL) are an initial feature of plaque

development. Circulating monocytes begin to accumulate in the area where they differentiate into macrophages and ingest oxLDL[51]. This gives rise to foam cells, the innermost cell type of an atherosclerotic plaque[52]. A progressed plaque consists of high numbers of inflammatory cells, enhanced extracellular matrix (ECM) degradation, excessive levels of cell apoptosis, and may eventually rupture, leading to thrombus formation and possible vessel occlusion.

3.1. Mast cells in atherosclerosis

The first to illustrate the importance of mast cells in atherosclerosis was Paris Constantinides in 1953, when he proposed a protective role for mast cells in rats, assigning this role to mast cell heparin[53]. Thereafter, a long silence in the field of mast cells and potential atherogenic mechanisms prevailed, with some frustration about the strong ability of mast cells to degrade and inactivate enzymes involved in lipoprotein metabolism. A renewed interest in the potential connection between mast cells and atherosclerosis was sparked when it was discovered that exocytosed granules of activated rat peritoneal mast cells were able to avidly degrade the apolipoprotein B-100 of LDL[54]. This initial observation led to the finding of activated mast cells being able to convert macrophages into foam cells, the hallmark of atherosclerosis[55]. Mast cells exert this effect *via* the "granule carrier pathway" in which the heparin and chymase components of the exocytosed mast cell granules act in concert. Thus, heparin first binds LDL particles, granule chymase then proteolyzes the bound particles and renders them unstable to fuse and form lipid droplets on granule surface, and ultimately macrophages phagocytoze the LDL-containing granules. This renewed interest in the mechanisms explaining the potential role of mast cells in atherosclerosis led to a search of mast cell proteases in the various layers of the healthy human arterial wall and in the various regions of atherosclerotic plaques. Indeed, it could be demonstrated that the mast cells in the human aortic wall are of two types, those containing only tryptase and those containing both tryptase and chymase[20]. Ever since numerous laboratories profoundly linked mast cells to the development of atherosclerosis and acute cardiovascular syndromes, such as atherothrombosis. In the human arterial wall, mast cells are located in the inner and outer layer (intima and adventitia) of a healthy aortic wall, first at a low density and then, during the progression of atherosclerosis, with a propensity to increased density in the vulnerable shoulder region of the developing plaque and also in the adventitia[20,56]. Most importantly, activated mast cells accumulate in the shoulder region of human coronary plaques, which is the site vulnerable to rupture[57]. In the atherosclerotic aortic wall of atherosclerosis-prone mice, mast cells are located mainly in the adventitia with only sporadic occurrence in the intima[58], while in humans they gather in both areas[56,59]. Moreover, mast cells in

the advanced atherosclerotic carotid and coronary arteries have been reported to be in close proximity to microvessel sprouting[59,60] and to be connected with intraplaque hemorrhage, *i.e.* with a key histological sign pointing to vulnerability of a plaque to rupture[61]. Notably, a recent publication on human carotid atherosclerosis described mast cells as the only immune cell type directly associated with future cardiovascular events[62]. Acquired evidence heretofore, recently reviewed by our group, appoints mast cells a pro-atherogenic role, as they have been reported to enhance leukocyte influx and local apoptosis, augment matrix degradation and induce hemorrhagic events, affecting plaque initiation, progression and destabilization[63].

3.2. Mast cell infiltration mechanisms

In the course of plaque development, mast cells infiltrate the vessels and are found in the intima and adventitia of human coronary arteries[64,65]. One mechanism of mast cell infiltration is *via* chemokine signals between eotaxin and its receptor C-C chemokine receptor-3[66]. However, mast cells carry additional chemokine receptors[67], which are also known to participate in transendothelial migration[68] during atherosclerosis development, indicating that there may be supplementary signals that attract mast cells to enter the arterial intima. Furthermore, activated mast cells can produce chemokines themselves[69], which may amplify the attraction signals and positively regulate their presence; possibly explaining the direct correlation between mast cell numbers and plaque progression. In addition, it has recently been reported that vascular endothelial cells (ECs) can induce immature mast cell attraction *via* the adhesion molecules E-selectin, P-selectin, vascular cell adhesion molecule-1 and platelet endothelial cell adhesion molecule-1[70]. Conversely, pro-inflammatory cytokines released by mast cells can induce upregulation of main adhesion molecules (e.g. P-selectin) in endothelial cells[71].

3.3. Mast cell activation mechanisms

It has been observed that apart from the numerical increase, mast cells appear in a degranulated state inside ruptured plaques of human coronary arteries[72], proposing therefore that mast cells exert their local effects through mediator release. Following that, we confirmed, using the atherosclerotic apoE-deficient ($apoE^{-/-}$) mouse model, that mast cells through their activation are responsible for disease progression, after observing an increase in plaque size upon systemic mast cell activation[73]. Concomitantly, Sun et al., (2007a) reported that double mutant LDL-receptor ($LDLr^{-/-}$)$Kit^{W\text{-}sh\backslash W\text{-}sh}$ mice show decreased plaque formation, lipid deposition and immune infiltration compared to the $LDLr^{-/-}$ strain, confirming a direct connection between mast cells and atherosclerosis.

Consequently, scientific research focused on possible target molecules that may activate mast cells inside the vascular microenvironment. Evidently, IgE was one of the primary candidate molecules to be investigated, with the first studies reporting that IgE serum levels were higher in patients suffering from angina pectoris[74] or dyslipidemia[75]. Moreover, IgE blood levels were detected to be significantly higher immediately after myocardial infarction[76]. Furthermore, in a recently published experimental study targeting immune activation in atherosclerosis, $LDLr^{-/-}$ mice treated with an antibody against OX40-ligand showed plaque regression which was partially appointed to reduced plasma IgE and lower mast cell infiltration[77]. Additionally, a few years ago it was stated that serum IgE could be detected within atherosclerotic s where it promoted cell apoptosis and cytokine expression in $apoE^{-/-}$ mice as well as in humans through FcεR1 in synchrony with Toll-like receptor-4 (TLR4)[78]. With respect to TLR4, despite the fact that the above effects were mainly described on macrophages, this receptor has also been found on the surface of mast cells where it was able to trigger membrane bound-mast cell activation[79], thus supporting a possible mast cell-TLR4 specific role in atherosclerosis. Moreover, antibodies of the IgG-class were found to activate human mast cells *in vitro*, after formation of immune complexes with oxLDL[80], indicating that various components of an immune reaction can contribute to intraplaque mast cell activation. Interestingly, oxLDL itself is one of the endogenous molecules that can initiate an immune response[81]. In relation to mast cells, oxLDL can directly activate human mast cells *in vitro*, partly through TLR-4 signaling[82]. In addition, oxLDL constituents, such as lysophosphatidic acid have lately been described to concentrate in the plaque and locally activate mast cells, contributing to plaque destabilization[83], and consequently illustrating a strong link between mast cell action and excessive lipid accumulation. Furthermore, components of the complement system, such as the anaphylatoxin C5a, are able to activate mast cells in the context of atherosclerosis through their specific C5a-receptor. Examination of human coronary plaques revealed that mast cells were positive for C5a-receptor[84]. A recent study directly linked C5a-specific mast cell activation in mice to plaque progression, showing that mast cell stabilization, using cromolyn, attenuated C5a-induced progression of vein graft disease, while mast cell activation using dinitrophenyl hapten augmented disease and increased plaque development[85]. Yet, apart from C5a, other proteins of the complement system may also act on mast cells inside the plaque microenvironment, since it has already been mentioned that certain mast cell subsets can get potently regulated by additional complement factors[86]. However, it is also the interaction with adjacent cell types inside the plaque that affects mast cell activation. For instance, ET-1, an endothelial vasoconstricting peptide, displays elevated levels in atherosclerosis patients[87] and induces cardiac mast cell activation in rats[88]. Furthermore, activated mast cells are able to enhance endothelial ET-1 expression *in vitro*[89]. Additionally, several bioactive molecules, such as reactive oxygen species,

present in the inflamed plaque at increased levels, may also function as local mast cell activators[90]. Lastly, an intriguing observation revealed that mast cells inside human coronary artery specimens of different atherosclerosis phases, reside proximal to nerve fibers[91], implicating the central nervous system in mast cell action. One link may be SP, a neuropeptide regulating inflammation[92] and stress-related cardiac syndromes[93]. Since mast cells were found to express the SP receptor, neurokinin-1[94], an experimental atherosclerosis mouse study was designed to identify the direct link between SP and these cells inside the plaque, revealing that mast cell presence and activation were enhanced upon intensified SP treatment, followed by plaque destabilization, while neurokinin-1 blockade prevented these changes[65]. Moreover, a very recent publication reported that an additional neurotransmitter, neuropeptide Y, can promote mast cell activation while also affecting plaque progression in apoE$^{-/-}$ mice[95]. Admittedly, there are probably other unidentified mast cell activators that elicit mediator release inside the plaque and further research needs to be conducted in order to identify the precise triggers of mast cell activation in atherosclerosis.

3.4 Mast cell mediated atherosclerosis progression

A vast amount of research has approached the role of mast cells in atherosclerosis by testing the effects of general mast cell inhibition achieved by pharmacological or genetic approaches. Among the evidence collected so far, it has been demonstrated that mast cells stabilized *in vitro*, using anti-allergic drugs, were able to inhibit foam cell formation, suggesting a direct role in plaque development[96]. In accordance, *in vivo* research, using the apoE$^{-/-}$Kit$^{W\text{-}sh/W\text{-}sh}$ mouse model, reports a reduction in aortic atherosclerosis progression, as well as leukocyte influx and inflammation levels[97]. Adding up to the above, a recent study using LDLr$^{-/-}$ mice, showed that mast cell stabilization with cromolyn not only reduced inflammation and atherosclerosis progression but also improved the lipid profile of these mice[98]. It is therefore evident, that mast cells, through their activation, can directly affect atherosclerosis progression.

However, as it may be expected, distinct mast cell activation pathways result in the release of diverse molecules, which individually affect the complex mechanisms of atherosclerosis in different aspects. Mast cell proteases are thought to be important in atherosclerosis (**Table 1**). For instance, chymase accounts for the disruption of vascular tissue homeostasis by indirect degradation of ECM *via* activation of pro-collagenase[99] and pro-matrix-metalloproteinase-9 (pro-MMP9)[100]. This serine protease can also directly degrade ECM by hydrolyzing its main components, such as fibronectin[101]. Furthermore, the direct degradation of fibronectin by chymase leads to vascular smooth muscle cell (VSMC) apoptosis[102]. Chymase can also induce VSMC apoptosis by disturbing the nuclear factor-kappa B (NF-κB) anti-apoptotic signaling pathway[103]. This

regulatory effect of mast cells, *via* chymase, on the VSMCs was recently found to be, at least partly, mediated through TLR-4 activation, in an *in vivo* atherosclerosis model[104]. Nevertheless, together with the induction of apoptosis, chymase can inhibit collagen production and hinder VSMC proliferation[105]. A combination of the above mechanisms can induce thinning of the fibrous cap of a plaque, and can lead to rupture. Moreover, this protease can also act on the vascular endothelium. Thus, chymase avidly degrades various components of the pericellular matrix of ECs, which may result in detachment of endothelial cells in turbulent areas of the arterial tree[106] and, in combination with TNFα, enhance EC apoptosis[107]. Chymase can also release latent TGF-β_1 from the pericellular matrix of ECs, and so render it susceptible for activation *via* interaction with binding sites on EC surface[108]. The activated TGF-β_1, again, can affect a multitude of signaling machineries involved in tissue remodeling, and it may also regulate the activity of infiltrating immune cells. Moreover, chymase is known to induce apoptosis of macrophages[73], while also stimulating immune effector cytokines and chemokines[109]. In addition, chymase has been demonstrated to degrade various apolipoproteins of high-density lipoprotein and thereby dampen the efflux of cholesterol from macrophage foam cells[110,111,112]. Also, experiments in the mouse have demonstrated that mast cell activation in the peritoneal cavity locally degrades apolipoprotein AI, and *via* this action impairs the entire macrophage reverse cholesterol transport pathway, *i.e.* cholesterol transport from the peritoneal macrophage foam cells to feces[111]. A number of studies report that chymase is responsible for the local production of Ang II[113,114]. More specifically, chymase-induced Ang II was able to induce reactive oxygen species production *via* binding to its receptor and activating NAD(P)H oxidase, aggravating hence the local inflammatory burden[115]. Subsequently, chymase expression, as well as Ang II levels, were found elevated in human atherosclerotic aortas[116], while, in a hamster model, Ang II levels were observed reduced after treatment with tranilast[117]. Ang II is also leading to VSMC apoptosis[118] and leukocyte infiltration[119]. A critical role for chymase, as a mediator for atherosclerosis progression, was demonstrated in apoE$^{-/-}$ mice in which this protease was inhibited, using compound RO5066852. These mice showed reduced plaque progression, increased plaque stability, through enhanced collagen deposition, and lower intraplaque hemorrhage levels[120].

Moreover, both chymase and tryptase were shown to be positively correlated with the degree of atherosclerosis in human aortas[121]. Tryptase is known to degrade ECM by activating pro-MMPs[122] while also breaking down fibronectin[106]. In addition, this protease has been suggested to actively participate in foam cell formation *in vitro*, through protease activated receptor-2 (PAR-2)[123], while enhancing MCP-1 and IL-8 levels on cultured endothelial cells[124]. Along that line, tryptase augments neutrophil migration by acting as an inducer of chemokine IL-8 secretion from endothelial cells[125]. Furthermore, tryptase acts on macrophages, by increasing lipid accumulation while

degrading high-density lipoprotein[126] and thus blocking the cholesterol efflux pathway. Yet, tryptase can affect various chemokines[127]. Additional evidence regarding the role of tryptase *per se* was gathered from apoE$^{-/-}$ mice that had recently undergone tryptase inhibition using lentiviral constructs. These mice presented increased angiogenesis and intraplaque hemorrhages[128].

Alongside with the protease secretion, inflammatory cytokines secreted by mast cells affect plaque phenotype. Human mast cells incubated with oxLDL-immune complexes *in vitro* were found to upregulate and secrete TNFα, Interleukin-8 and macrophage chemoattractant protein-1[80]. Moreover, TNFα, together with IL-6 and IFNγ, secreted by mast cells were able to regulate *in vitro* the expression of endothelial cell adhesion molecules[71]. Interestingly, Sun et al. (2007a) reported that mast cell specific IL-6 and IFNγ, but not TNFα, cytokine secretion can promote atherosclerosis *in vivo* upon induction of cathepsin activation and ECM degradation[58]. Furthermore, mast cells inside human coronary artery plaques were also capable of producing basic fibroblast growth factor, intensifying the link with intraplaque angiogenesis[61]. Finally, additional mast cell specific mediators such as histamine and heparin have been also reported to participate in mast cell mediated atherosclerosis progression; by inducing macrophage apoptosis[73] or enhancing foam cell formation[55] respectively.

Thereupon, the effects of mast cell activation are induced through the differential action of their degranulated mediators. which act in the surrounding microenvironment and alter the plaque phenotype either by increasing plaque size or by diminishing plaque stability. For that reason, a considerable amount of research focuses on the inhibition of specific mast cell activation pathways in an attempt to hamper atherosclerosis progression while still retaining beneficial mast cell effector functions, for example as components of the host-immune response.

Chymase	Tryptase
Breakdown of ECM components	Breakdown of ECM components
Vascular SMC apoptosis	PAR-2 activation
Collagen degradation	Increased lipid accumulation
Disruption of vascular EC layer	Disruption of vascular EC layer
Vascular EC apoptosis	Macrophage apoptosis
Induction of leukocyte infiltration in the subendothelial space	Induction of leukocyte infiltration in the subendothelial space
HDL and LDLproteolysis	HDL proteolysis
Cytokine/chemokine regulation	Cytokine/chemokine regulation
Macrophage apoptosis	Angiogenesis
Vascular Ang II production	Immunoglobulin synthesis

Table 1: Chymase and tryptase effects in atherosclerosis. *ECM: extracellular matrix, SMC: smooth muscle cells, EC: endothelial cells, Ang II: angiotensin II, ROS: reactive oxygen species, PAR-2: protease activated receptor-2, PAF: platelet activating factor, HDL: high-density lipoprotein; LDL: low-density lipoprotein.*

4. Restenosis

The available surgical intervention methods, used so far, to treat an atherosclerotic artery are bypass surgery, balloon angioplasty and stent placement; however, this often leads to restenosis[129]. The major differences between a restenotic and an atherosclerotic plaque are primarily the acute rate of inflammation and neointima formation induced by endothelial damage, as a consequence of the therapeutic intervention, as well as increased fibrosis caused by enhanced VSMC proliferation[130].

Seemingly, since mast cells have already infiltrated the vessels upon primary atherosclerosis development, restenotic plaques contain an increased amount of mast cells ready to degranulate upon trigger[131], with their mediators aggravating the restenotic burden. In particular, chymase-generated Ang II and TGF-β_1 are linked to fibroblast proliferation and neointimal formation[132]. Therefore, a general Ang II receptor inhibitor used in an experimental model of dog angioplasty showed reduced neointima development[133], an effect partly caused by blockade of chymase-induced Ang II. Moreover, tranilast used on dog carotid arteries after balloon injury was able to suppress chymase expression and hinder neointimal formation[131]. Tryptase is also implicated in restenosis, mainly through PAR-2 activation, affecting transendothelial leukocyte infiltration. More specifically, PAR-2 deficient mice showed reduced leukocyte adhesion in the endothelial wall which contributed to reduced neointima size[134]. In the same manner as Ang II, PAR-2 effects are partly linked to tryptase-induced activation.

Another mast cell mediator which has attracted attention through the years in restenosis is histamine, with prior *in vitro* data suggesting that it plays a role in EC and SMC proliferation and migration[135]. An *in vivo* study in which the histamine receptor H1 was inhibited in mice suffering endothelial damage after coronary angioplasty, reported diminished neointima size and reduced cell proliferation inside the plaque[133]. In line with that, *in vivo* data from pigs that underwent stent placement revealed that local histamine concentration is increasing over time[136]. In contrast to the above mentioned *in vitro* study the authors did not observe any histamine-triggered proliferation in cultured SMCs hetherto. Nonetheless, it is important to note that histamine effects should not be entirely ascribed to mast cells since macrophages[137] and platelets[138] can show inducible histamine production as well.

4.1 Drug Eluting Stents

In order to avoid the excessive VSMC proliferation that follows stent or angioplasty-generated trauma, Drug Eluting Stents (DES), containing specific anti-proliferative drugs, have been developed[139]. With regard to the role of mast cells in DES,

a very recent study proposed nitric oxide and ROS-scavenger coated stents as beneficial agents in mast cell stabilization inside the implanted artery[140].

Interestingly, a striking effect that highlights the importance of mast cells in acute coronary events is described under the name Kounis syndrome[141], in which hypersensitivity reactions, such as anaphylaxis, can occur upon DES placement and develop in a synchronized manner with acute coronary events. Recent studies have revealed that DES coating elements such as metal, latex and polymer, as well as the eluted drugs, can cause hypersensitivity reactions[142]. It is therefore suggested that mast cell activation may lead to increased inflammation, and possible thrombosis, in an acute fashion inside a restenotic environment, underlining the influential effect of these cells in yet another cardiovascular syndrome.

5. Myocardial infarction

The clinical outcome of a ruptured atherosclerotic plaque is mostly induced by the formation of a thrombus, which can destabilize upon high blood flow and relocate in smaller vessels, obstructing the circulation. Progressed stenotic plaques inside a coronary artery often lead to the occlusion of vessels that supply the heart muscle with blood, giving rise to an acute myocardial infarction (MI)[143].

Mast cells have been directly linked to plaque rupture, since they accumulate in the shoulder region of ruptured atherosclerotic plaques of patients that have suffered an MI[57]. Similarly, analysis of human coronary specimens after MI, displayed increased numbers of degranulated mast cells in the adventitia of ruptured plaques[64]. These cells were mainly positive for chymase, histamine and tryptase.

Consequently, attention turned to mast cell specific proteins in MI, with the first data coming from infarcted cardiac tissues in a hamster model. This study in hamsters presented increased chymase activity in the ischemic area, from day 1 and until day 56 post-MI, while chymase positive mast cell numbers were found elevated, particularly in the initial phase of the MI[144]. Moreover, chymase-mediated Ang II production was studied. Specifically, systemic Ang II production was blocked using an ACE blocker and the subsequent effects were compared to the overall Ang II effects obtained upon using an Ang II receptor inhibitor. This experiment showed a decrease in the mortality rate only upon Ang II blockade, suggesting indirectly that chymase-induced Ang II production is responsible for the detrimental effects in the ischemic tissue following an MI. A follow-up study from the same group, in which chymase was blocked in hamsters after mechanical MI induction, reported improved cardiac function and decreased mortality rates, highlighting the importance of this protease in infarcted tissues[145]. Therefore, from a therapeutic view, it has been proposed that chymase inhibitors

can significantly improve cardiac function upon combination with general Ang II inhibitors[146]. Nevertheless, the effects of chymase appear to be caused not only *via* Ang II but also partly *via* TGF-β. Chymase inhibition in rats showed reduced TGF-β expression in combination with reduced myocardial fibrosis levels and cardiac dysfunction[147]. Lastly, aside from TGF-β, chymase may additionally exert its effects through activation of MMP-9, since its inhibition in pigs resulted in lower levels of activated MMP-9 which was accompanied by a lower infarct size, as well as reduced inflammation and apoptosis levels[148].

Along this line, experiments using the mast cell inhibitor relaxin in a swine MI model have confirmed a reduction in the plasma histamine, together with a decrease in mast cell degranulation[149]; but also lower cardiac tissue injury. This points towards a role for histamine in MI, while highlighting the importance of mast cells in the pathophysiology of the ischemic tissue. In addition, upon examination of inflammatory levels in human serum after an acute MI event, MCP-1 was found significantly increased and positively correlated to IL-8[150], which may illustrate a partial implication of mast cells, since they can secrete both cytokines.

Interestingly, research on late-phase infarcted heart tissues in dogs suggested that tryptase contributes to the upregulation of MCP-1 and IL-8 in the cardiac endothelium, thereby leading to increased angiogenesis and promotion of the healing process in the ischemic myocardium[151]. Similarly, a recent study in infarcted rat hearts locally treated with mast cell granules isolated from rat peritoneal cells, demonstrated reduced cardiomyocyte apoptosis, increased angiogenesis levels and improved cardiac function[152], providing evidence in favor of a cardioprotective role exerted by mast cell granule contents. Thereupon, mast cells through their diverse mediator effects seem to participate not only in the generation of an MI, but also in the cardiac tissue recovery following it.

6. Arrhythmia

Atherosclerosis and MI are both linked to certain types of cardiac arrhythmia[153], a condition during which the myocardial rhythm appears disrupted as a result of irregular electrical activity. One of the classification methods for distinct types of arrhythmia is based on the location where the dysfunction occurs, with characteristic examples atrial and ventricular arrhythmias[154]. Atrial fibrillation, the most common type of arrhythmia, is tightly connected to pre-established atherosclerosis, with the latest study directly associating human carotid intima/media thickness with the occurrence of this type of tachycardia[155]. On the other hand, ventricular arrhythmias are frequently described as a result of an MI incident[156] upon over-excitation of cardiac nerve fibers.

Clearly, since mast cells are associated with both atherosclerosis and MI, attention was also drawn to their possible implication in cardiac arrhythmias. The primary indications in favor of such a relationship were collected in a 1986 review which scrutinized the arrhythmogenic effects of histamine in pivotal sectors of the heart muscle[157]. The role of histamine was explored in detail after induction of MI, in rodent and canine models, demonstrating that the presence of histamine is directly associated with post-MI generation of ventricular arrhythmias[158]; and moreover, that its concentration increases proportionally to the severity of the ischemic incident[159]. The mast cell inhibitor relaxin was able to lower histamine content in the heart and reduce ventricular tachycardia, expanding thus the beneficial outcome of mast cell stabilization in arrhythmias[149]. Histamine exerts its function through a series of G-coupled protein receptors (H_1-H_4) which may exhibit differential downstream actions. For instance, while H_2 receptor is reported to show an arrhythmogenic function[160], H_3 receptor is considered as benefactor for the alleviation of post MI-tachyarrhythmic events[161]. To make matters even more complex, it has recently been suggested that mast cells are not the only source of cardiac histamine, and therefore not the only cell type responsible for the ventricular arrhythmias observed after an MI incident[162]. Nonetheless, the majority of evidence so far highlights the detrimental role of mast cells in cardiac arrhythmias, which appears to be mediated through release of their mediators. For example, after blockade of chymase in an infarcted dog model, ventricular arrhythmias as well as serum Ang II levels were reduced[163], implicating thus chymase release and local Ang II formation in the generation of post-MI tachycardia. Furthermore, SP was reported to trigger renin release by cardiac mast cells in *ex vivo* ischemia experiments, with the latter further enhancing arrhythmogenicity[164,165].

Finally, recently obtained data connect mast cells not only to arrhythmias following pre-established cardiac disorders, but also to newly generated atrial fibrillation events which can subsequently lead to acute cardiovascular syndromes. Specifically, in a mouse study of atrial fibrillation, less fibrosis in the atrium was observed after cromolyn stabilization or genetic reconstitution with mast cell deficient $Kit^{W/W\text{-}v}$ bone marrow. This was appointed to a cross-talk mechanism between cardiac mast cells and myocytes or fibroblasts, through platelet-derived growth factor-A[166]. Based on the above, it is evident that the impact of mast cells in this cardiac disease is not entirely understood, since it has begun to attract full attention only recently.

7. Aneurysm

The development of an aneurysm is the consequence of vascular wall thinning, following accelerated smooth muscle cell death and increased ECM breakdown, accompanied by increased levels of inflammation, occurring for example in the abdominal

aorta (abdominal aortic aneurysm or AAA) or in the brain (cerebral aneurysm)[167]. Following these events, a vessel can rupture, leading to excessive hemorrhage and strongly increased risk of death[168].

7.1 Cerebral aneurysm

Among research linking mast cells to aneurysm formation, a distinctive study, on humans who suffered a cerebral aneurysm rupture, reported mast cells at increased numbers near ruptured vessels[169]. In another study, treatment of rats with mast cell inhibitors reduced macrophage infiltration and vessel thickening, and mast cells were proposed to induce the expression and activation of pro-MMPs in adjacent SMCs[170]. Furthermore, a report on human tissue specimens confirmed that mast cells, together with inflammatory macrophages, are abundant in ruptured aneurysms[171]. Lastly, another study demonstrated that mast cells which reside within the thin wall of a remodeled and eroded human intracranial aneurysm, were located in close proximity to newly formed microvessels and associated with micro-hemorrhages[172]. These observations suggested that mast cells contribute to the wall remodeling and degeneration in the small intracranial arteries which develop saccular aneurysms and may ultimately rupture.

7.2 Abdominal aortic aneurysm

The strongest evidence on the role of mast cells in aneurysms is obtained from abdominal aortic aneurysms[21]. Regarding the recruitment of mast cells inside the aneurysmal area, a study revealed that transplantation of mast cells from $apoE^{-/-}$ mice with a defect in the MCP-1 receptor, CCR2, in $apoE^{-/-}Kit^{W\text{-}sh/W\text{-}sh}$ mice reduced aortic aneurysm formation, in comparison to the $apoE^{-/-}$ strain, as a result of decreased mast cell presence; proposing a possible CCR2-mediated signaling pathway for the migration of mast cells inside the diseased vessel wall[173].

In terms of mast cell action in aneurysms, the impact of IL-6 and IFNγ has also been established in aneurysm formation, and reconstitution experiments in the $Kit^{W\text{-}sh/W\text{-}sh}$ mice have proven that these cytokines play a fundamental role in the induction of aneurysms[174]. In addition, mast cell deficient mice were protected from aneurysms, presenting decreased leukocyte infiltration in the injured area, as well as reduced levels of apoptosis and angiogenesis; suggesting thus that mast cells are important effectors in these mechanistic changes inside the diseased vessel.

Furthermore, one of the above mentioned studies described increased Ang II formation in aneurysmal and atherosclerotic aortic samples, assigning chymase an

important role in both syndromes[111]. The most remarkable observation in this study was the increased numbers of activated mast cells in aneurysmal aortas, which were even higher than in atherosclerotic samples.

The effect of chymase in this disease was also underlined in an experimental hamster model in which aneurysm formation was inhibited upon treatment with the chymase inhibitor, NK3201[175]. Additional evidence implicates chymase in the progression of aneurysm formation, as high levels of chymase-positive mast cells in human aortas directly associate with the development of the disease[176]. This study also provided a causal relation between mast cells and aneurysm formation, by showing reduced aneurysm incidence in both chymase deficient and $Kit^{W\text{-}sh/W\text{-}sh}$ mice, but also after reconstitution of the $Kit^{W\text{-}sh/W\text{-}sh}$ mice with chymase deficient mast cells. The authors suggested that chymase exerts its function through cathepsin expression, ECM degradation and angiogenesis.

Yet, once more, tryptase was also proven to directly participate in the development of abdominal aneurysms, after lack of experimental aneurysm formation in tryptase-deficient mice[177]. These observations were again combined with data acquired from human aortic samples, showing tryptase accumulation inside aneurysmal tissues while its circulatory levels directly correlated with aneurysm progression rate. Similarly to atherosclerosis, aortic aneurysms display increased expression of chymase, tryptase and cathepsin-G, together with angiogenic proteins expression levels and with the number of adventitial mast cells, all correlating with the degree of neovascularization[178]. The critical influence of mast cells has also been established after chemical stabilization with tranilast in $apoE^{-/-}$ mice and mast cell deficient rats, with both models presenting a reduction in aneurysm formation[179].

Finally, a mouse study uncovered a role for IgE in abdominal aortic aneurysms, showing aggravated disease progression by IgE mediated activation of not only mast cells, but also T cells and macrophages[180]. Moreover, FcεR1-deficient $apoE^{-/-}$ mice failed to develop abdominal aneurysms and presented lower plasma IL-6 levels[180]. With reference to this study, it is important to note that although FcεR-mediated effects are generally considered mast cell specific, increasing evidence supports the notion of an inducible FcεR-expression pattern in additional immune populations, such as T cells and macrophages, depending on the environmental stimuli, emphasizing thus the plastic nature of disease-specific immune responses.

An example of this plasticity comes from a study examining the role of the peptide hormone adrenomedullin in human abdominal aortic aneurysms, and revealing that this peptide can be released from mast cells and act as an anti-fibrotic agent[181]. These results contradict the previously discussed pro-fibrotic role of mast cells in aneurysm

formation, and illustrate the diverse, and possible immune-regulatory, responses of this immune-effector cell type upon excessive inflammation levels.

8. Therapeutic potential

Mast cells are rightly regarded as potent inflammatory cells in cardiovascular diseases. The massive amount of evidence gathered so far mainly relates to their harmful pathophysiological events, thereby leading to the perception that global inhibition of mast cell activation could save the local microenvironment from their disadvantageous effects. For that very reason, as well as to examine the overall influence of mast cells on disease progression, general mast cell inhibitors, such as cromolyn, are widely used in experimental models; mainly suggesting that mast cell stabilization can mitigate the inflammation levels in acute cardiovascular syndromes. Furthermore, pharmacological mast cell stabilizers, already widely used in allergic disorders, were considered as promising therapeutic agents in humans suffering from vascular diseases, a characteristic example being the use of tranilast in a large patient study for the prevention of restenosis; without however presenting any beneficial clinical outcome[182]. The adverse effects of this application can be predicted by recognizing that mast cells do not only reside in the heart and vessels, but also comprise the first line of host defense against pathogens. Therefore, a possible systemic inhibition may compromise the protective responses exerted by mast cells as part of immune protection.

Therefore, new technologies are applied, in order to scan for novel targeting molecules that may avert from the risks of systemic mast cell inhibition[183]. An appealing alternative would be to focus on the type and magnitude of the differential activation and inhibition signals, to manage the sort of mast cell reaction needed per occasion[184]. In an attempt to concentrate on the action of specific mast cell mediators, rather than the general activation pathway, chemical inhibitors of serine proteases and histamine receptor antagonists have been designed and tested in animal models, and they have, indeed showed some very promising results[185]. However, the inhibition of molecules with such a broad spectrum of effects can also increase the risk of hindering multiple downstream mechanisms, not all of them being harmful. For instance, both proteases are able to degrade pro-inflammatory cytokines[186], probably upon highly inflammatory conditions, an effect which may prove beneficial. Thus, elucidation of the net effect, beneficial or harmful, in such highly complex *in vivo* conditions requires rigorous experimental testing in suitable animal models of the disease conditions in question.

Mast cells have also been described to be important mediators in immune suppression. An intriguing paradigm is an allograft study in which these cells were directed to enter tolerant grafts, after responding to high IL-9 levels secreted by T

regulatory lymphocytes[187]. In favor of this immune regulatory role, mast cells have been found to possess an interesting ability to communicate with distant targets *via* secretion of small membrane vesicles containing cytokines and other mediators, named exosomes and microparticles[188]. These vesicles are proven to carry molecules *via* which they elicit immune-regulatory mechanisms[189]; suggesting that enhancement of immune tolerance, rather than chemical inhibition of mast cell activation, may prove more advantageous as a therapeutic method.

The above illustrated examples on the importance, as well as the complexity of mast cells, suggest that manipulation of this cell type should be approached in a delicate way, superseding the general consideration of them being detrimental effector cells that need to be blocked at any cost. After all, it is not by accident that a very recent review on the field of cancer immunology has addressed mast cells as "sentinel cells" that may be alternatively modulated[190], rather than inhibited, in order to provide beneficial immune reactions and contribute to improved prognosis.

9. Conclusion

This review aimed to highlight the potential effects of mast cells in cardiovascular diseases, which are characterized by increased morbidity and mortality rates worldwide. In conclusion, mast cells can be considered to be important effector cells of the immune system, acting as a driving force of the local response, either by their direct cell-cell interaction with adjacent vascular cell types (**Figure 2**), or *via* their mediator release; resulting in targeted stimulation of cells residing in the microenvironment in which the mast cells have been activated. The plethora of secreted molecules, together with the multiple activation pathways, allows the identification of specialized therapeutic interventions focusing on the distinct features of each disease, while refraining from interfering with their beneficial characteristics. Therefore, modulation of individual pathways in mast cell function, rather than systemic mast cell blockade, may serve as a promising strategy to alleviate the progression of cardiovascular diseases, with an ultimate aim to reduce the excess morbidity and mortality associated with these disorders.

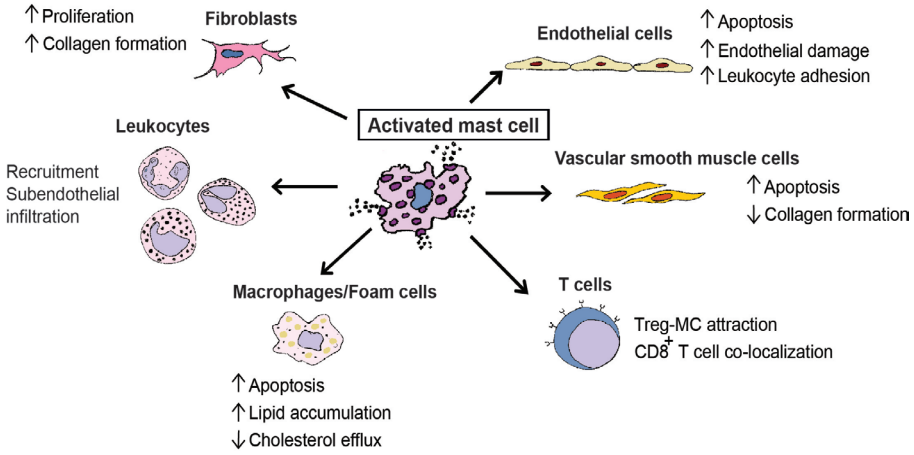


Figure 2: Mast cell interaction with adjacent cell types in the vasculature. This schematic overview illustrates the major cell interaction mechanisms between mast cells and various cell types present in the vessels. Activated mast cells are able to affect key biological processes, such as proliferation and apoptosis, of resident cell types in the cardiovascular system.

Reference list:

1. Beaven, M. A. Our perception of the mast cell from Paul Ehrlich to now. *Eur. J. Immunol.* **39,** 11–25 (2009).
2. Kitamura, Y. Heterogeneity of mast cells and phenotypic change between subpopulations. *Annu. Rev. Immunol.* **7,** 59–76 (1989).
3. Dahlin, J. S. & Hallgren, J. Mast cell progenitors: Origin, development and migration to tissues. *Molecular Immunology* **63,** 9–17 (2015).
4. Kraft, S. & Kinet, J.-P. New developments in FcepsilonRI regulation, function and inhibition. *Nature reviews. Immunology* **7,** 365–378 (2007).
5. Woolhiser, M. R., Okayama, Y., Gilfillan, a M. & Metcalfe, D. D. IgG-dependent activation of human mast cells following up-regulation of FcgammaRI by IFN-gamma. *Eur. J. Immunol.* **31,** 3298–3307 (2001).
6. Erdei, A., Andrásfalvy, M., Péterfy, H., Tóth, G. & Pecht, I. Regulation of mast cell activation by complement-derived peptides. in *Immunology Letters* **92,** 39–42 (2004).
7. Varadaradjalou, S. *et al.* Toll-like receptor 2 (TLR2) and TLR4 differentially activate human mast cells. *Eur J Immunol* **33,** 899–906 (2003).
8. Li, W.-W. *et al.* Substance P Signaling Controls Mast Cell Activation, Degranulation, and Nociceptive Sensitization in a Rat Fracture Model of Complex Regional Pain Syndrome. *Anesthesiology* **116,** 882–895 (2012).
9. Szalay, L., Kaszaki, J., Nagy, S. & Boros, M. Endothelin-1 induces mucosal mast cell degranulation in the rat small intestine. *Life Sci.* **67,** 1947–58 (2000).
10. Wernersson, S. & Pejler, G. Mast cell secretory granules: armed for battle. *Nat. Rev. Immunol.* **14,** 478–494 (2014).
11. Friend, D. S. *et al.* Mast cells that reside at different locations in the jejunum of mice infected with Trichinella spiralis exhibit sequential changes in their granule ultrastructure and chymase phenotype. *J. Cell Biol.* **135,** 279–290 (1996).
12. Hsieh, F. H. *et al.* Human airway epithelial cell determinants of survival and functional phenotype for primary human mast cells. *Proc. Natl. Acad. Sci.* **102,** 14380–14385 (2005).
13. Gordon, J. R. & Galli, S. J. Release of both preformed and newly synthesized tumor necrosis factor alpha (TNF-alpha)/cachectin by mouse mast cells stimulated via the Fc epsilon RI. A mechanism for the sustained action of mast cell-derived TNF-alpha during IgE-dependent biological res. *J. Exp. Med.* **174,** 103–107 (1991).
14. Galli, S. J. *et al.* Mast cells as "tunable" effector and immunoregulatory cells: recent advances. *Annu. Rev. Immunol.* **23,** 749–86 (2005).
15. Rakusan, K., Sarkar, K., Turek, Z. & Wicker, P. Mast cells in the rat heart during normal growth and in cardiac hypertrophy. *Circ. Res.* **66,** 511–516 (1990).
16. Silver, R. B. *et al.* Mast cells: a unique source of renin. *Proc Natl Acad Sci U S A* **101,** 13607–13612 (2004).
17. Balakumar, P. *et al.* Resident cardiac mast cells: Are they the major culprit in the pathogenesis of cardiac hypertrophy? *Basic and Clinical Pharmacology and Toxicology* **102,** 5–9 (2008).
18. Kennedy, S., Wu, J., Wadsworth, R. M., Lawrence, C. E. & Maffia, P. Mast cells and vascular diseases. *Pharmacol Ther* **138,** 53–65 (2013).
19. Kitamura, Y., Oboki, K. & Ito, A. Development of mast cells. *Proc. Jpn. Acad. Ser. B. Phys. Biol. Sci.* **83,** 164–74 (2007).
20. Kaartinen, M., Penttila, A. & Kovanen, P. T. Mast cells of two types differing in neutral protease composition in the human aortic intima. Demonstration of tryptase- and tryptase/chymase-containing mast cells in normal intimas, fatty streaks, and the shoulder region of atheromas. *Arterioscler. Thromb. a J. Vasc. Biol.* **14,** 966–972 (1994).

21. Swedenborg, J., Mäyränpää, M. I. & Kovanen, P. T. Mast cells: Important players in the orchestrated pathogenesis of abdominal aortic aneurysms. *Arterioscler. Thromb. Vasc. Biol.* **31,** 734–740 (2011).
22. Levick, S. P. *et al.* Cardiac mast cells: The centrepiece in adverse myocardial remodelling. *Cardiovascular Research* **89,** 12–19 (2011).
23. Bot, I. & Biessen, E. A. L. Mast cells in atherosclerosis. *Thromb. Haemost.* **106,** 820–826 (2011).
24. Fuster, V., Kelly, B. B. & Vedanthan, R. Global cardiovascular health: Urgent need for an intersectoral approach. *J. Am. Coll. Cardiol.* **58,** 1208–1210 (2011).
25. Bray, G. A. & Bellanger, T. Epidemiology, Trends, and Morbidities of Obesity and the Metabolic Syndrome. *Endocrine* **29,** 109–118 (2006).
26. Weisberg, S. P. *et al.* Obesity is associated with macrophage accumulation in adipose tissue. *J. Clin. Invest.* **112,** 1796–1808 (2003).
27. Boulet, N., Estève, D., Bouloumié, A. & Galitzky, J. Cellular heterogeneity in superficial and deep subcutaneous adipose tissues in overweight patients. *J. Physiol. Biochem.* **69,** 575–583 (2013).
28. Xu, J.-M. & Shi, G.-P. Emerging Role of Mast Cells and Macrophages in Cardiovascular and Metabolic Diseases. *Endocr. Rev.* **33,** 71–108 (2012).
29. Tanaka, A., Nomura, Y., Matsuda, A., Ohmori, K. & Matsuda, H. Mast cells function as an alternative modulator of adipogenesis through 15-deoxy-delta-12, 14-prostaglandin J2. *AJP Cell Physiol.* **301,** C1360–C1367 (2011).
30. Cao, H. Adipocytokines in obesity and metabolic disease. *J. Endocrinol.* **220,** T47-59 (2014).
31. Sismanopoulos, N. *et al.* Do mast cells link obesity and asthma? *Allergy: European Journal of Allergy and Clinical Immunology* **68,** 8–15 (2013).
32. Ervin, R. B. Prevalence of metabolic syndrome among adults 20 years of age and over, by sex, age, race and ethnicity, and body mass index: United States, 2003-2006. *Natl. Health Stat. Report.* 1–7 (2009). at <http://www.ncbi.nlm.nih.gov/pubmed/19634296>
33. Liu, J. *et al.* Genetic deficiency and pharmacological stabilization of mast cells reduce diet-induced obesity and diabetes in mice. *Nat. Med.* **15,** 940–945 (2009).
34. Hotamisligil, G. S., Shargill, N. S. & Spiegelman, B. M. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science (80-.).* **259,** 87–91 (1993).
35. Feinstein, R., Kanety, H., Papa, M. Z., Lunenfeld, B. & Karasik, A. Tumor necrosis factor-alpha suppresses insulin-induced tyrosine phosphorylation of insulin receptor and its substrates. *J Biol Chem* **268,** 26055–26058 (1993).
36. Gonzalez Suarez, M. L., Thomas, D. B., Barisoni, L. & Fornoni, A. Diabetic nephropathy: Is it time yet for routine kidney biopsy? *World J. Diabetes* **4,** 245–55 (2013).
37. Waanders, F., Visser, F. W. & Gans, R. O. B. Current concepts in the management of diabetic nephropathy. *Neth. J. Med.* **71,** 448–58 (2013).
38. Ruger, B. M. *et al.* Mast cells and type VIII collagen in human diabetic nephropathy. *Diabetologia* **39,** 1215–1222 (1996).
39. Hiromura, K., Kurosawa, M., Yano, S. & Naruse, T. Tubulointerstitial mast cell infiltration in glomerulonephritis. *Am. J. Kidney Dis.* **32,** 593–599 (1998).
40. Jones, S. E. *et al.* Mast cell infiltration and chemokine expression in progressive renal disease. *Kidney Int.* **64,** 906–913 (2003).
41. Yu, L., Border, W. A., Huang, Y. & Noble, N. A. TGF-β isoforms in renal fibrogenesis. *Kidney Int.* **64,** 844–856 (2003).
42. Rodrigues-Diez, R. *et al.* Gremlin is a downstream profibrotic mediator of transforming

growth factor-beta in cultured renal cells. *Nephron - Exp. Nephrol.* **122,** 62–74 (2013).

43. Wasse, H., Naqvi, N. & Husain, A. Impact of Mast Cell Chymase on Renal Disease Progression. *Curr. Hypertens. Rev.* **8,** 15–23 (2012).
44. Prasad, P. *et al.* Chronic renal insufficiency among Asian Indians with type 2 diabetes: I. Role of RAAS gene polymorphisms. *BMC Med Genet* **7,** 42 (2006).
45. Kondo, S. *et al.* Role of mast cell tryptase in renal interstitial fibrosis. *J. Am. Soc. Nephrol.* **12,** 1668–1676 (2001).
46. Jones, S. E., Gilbert, R. E. & Kelly, D. J. Tranilast reduces mesenteric vascular collagen deposition and chymase-positive mast cells in experimental diabetes. *J. Diabetes Complications* **18,** 309–315 (2004).
47. Huang, Z. G. *et al.* Myocardial Remodeling in Diabetic Cardiomyopathy Associated with Cardiac Mast Cell Activation. *PLoS One* **8,** (2013).
48. Moreno, M. *et al.* Circulating tryptase as a marker for subclinical atherosclerosis in obese subjects. *PLoS One* **9,** (2014).
49. Go, A. S. *et al.* Heart disease and stroke statistics--2014 update: a report from the American Heart Association. *Circulation* **129,** e28–e292 (2014).
50. Libby, P., Ridker, P. M. & Hansson, G. K. Progress and challenges in translating the biology of atherosclerosis. *Nature* **473,** 317–325 (2011).
51. Tsimikas, S. & Miller, Y. I. Oxidative modification of lipoproteins: mechanisms, role in inflammation and potential clinical applications in cardiovascular disease. *Curr Pharm Des* **17,** 27–37 (2011).
52. Rader, D. J. & Puré, E. Lipoproteins, macrophage function, and atherosclerosis: Beyond the foam cell? *Cell Metabolism* **1,** 223–230 (2005).
53. Constantinides, P. Mast cells and susceptibility to experimental atherosclerosis. *Science* **117,** 505–6 (1953).
54. Kokkonen, J. O. & Kovanen, P. T. Low density lipoprotein degradation by rat mast cells. Demonstration of extracellular proteolysis caused by mast cell granules. *J. Biol. Chem.* **260,** 14756–14763 (1985).
55. Kokkonen, J. O. & Kovanen, P. T. Stimulation of mast cells leads to cholesterol accumulation in macrophages in vitro by a mast cell granule-mediated uptake of low density lipoprotein. *Proc. Natl. Acad. Sci. U. S. A.* **84,** 2287–91 (1987).
56. Atkinson, J. B., Harlan, C. W., Harlan, G. C. & Virmani, R. The association of mast cells and atherosclerosis: A morphologic study of early atherosclerotic lesions in young people. *Hum. Pathol.* **25,** 154–159 (1994).
57. Kaartinen, M., Penttila, a. & Kovanen, P. T. Accumulation of activated mast cells in the shoulder region of human coronary atheroma, the predilection site of atheromatous rupture. *Circulation* **90,** 1669–1678 (1994).
58. Sun, J. *et al.* Mast cells promote atherosclerosis by releasing proinflammatory cytokines. *Nat. Med.* **13,** 719–724 (2007).
59. Jeziorska, M., McCollum, C. & Woolley, D. E. Mast cell distribution, activation, and phenotype in atherosclerotic lesions of human carotid arteries. *J. Pathol.* **182,** 115–22 (1997).
60. Kaartinen, M., Penttila, A. & Kovanen, P. T. Mast cells accompany microvessels in human coronary atheromas: implications for intimal neovascularization and hemorrhage. *Atherosclerosis* **123,** 123–131 (1996).
61. Lappalainen, H., Laine, P., Pentikäinen, M. O., Sajantila, A. & Kovanen, P. T. Mast cells in neovascularized human coronary plaques store and secrete basic fibroblast growth factor, a potent angiogenic mediator. *Arterioscler. Thromb. Vasc. Biol.* **24,** 1880–1885 (2004).

62. Willems, S. *et al.* Mast cells in human carotid atherosclerotic plaques are associated with intraplaque microvessel density and the occurrence of future cardiovascular events. *Eur. Heart J.* **34,** 3699–3706 (2013).
63. Bot, I., Shi, G.-P. & Kovanen, P. T. Mast cells as effectors in atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* **35,** 265–271 (2015).
64. Laine, P. *et al.* Association Between Myocardial Infarction and the Mast Cells in the Adventitia of the Infarct-Related Coronary Artery. *Circulation* **99,** 361–369 (1999).
65. Bot, I. *et al.* The neuropeptide substance P mediates adventitial mast cell activation and induces intraplaque hemorrhage in advanced atherosclerosis. *Circ. Res.* **106,** 89–92 (2010).
66. Haley, K. J. *et al.* Overexpression of eotaxin and the CCR3 receptor in human atherosclerosis: using genomic technology to identify a potential novel pathway of vascular inflammation. *Circulation* **102,** 2185–9 (2000).
67. Juremalm, M. & Nilsson, G. Chemokine receptor expression by mast cells. *Chem Immunol Allergy* **87,** 130–144 (2005).
68. Zernecke, A., Shagdarsuren, E. & Weber, C. Chemokines in atherosclerosis an update. *Arteriosclerosis, Thrombosis, and Vascular Biology* **28,** 1897–1908 (2008).
69. Oliveira, S. H. P. & Lukacs, N. W. Stem cell factor and IgE-stimulated murine mast cells produce chemokines (CCL2, CCL17, CCL22) and express chemokine receptors. *Inflamm. Res.* **50,** 168–174 (2001).
70. Dudeck, A. *et al.* Immature mast cells exhibit rolling and adhesion to endothelial cells and subsequent diapedesis triggered by E- and P-selectin, VCAM-1 and PECAM-1. *Exp. Dermatol.* **19,** 424–434 (2010).
71. Zhang, J. *et al.* Regulation of endothelial cell adhesion molecule expression by mast cells, macrophages, and neutrophils. *PLoS One* **6,** (2011).
72. Kovanen, P. T., Kaartinen, M. & Paavonen, T. Infiltrates of Activated Mast Cells at the Site of Coronary Atheromatous Erosion or Rupture in Myocardial Infarction. *Circulation* **92,** 1084–1088 (1995).
73. Bot, I. *et al.* Perivascular mast cells promote atherogenesis and induce plaque destabilization in apolipoprotein E-deficient mice. *Circulation* **115,** 2516–25 (2007).
74. Korkmaz, M. E. *et al.* Levels of IgE in the serum of patients with coronary arterial disease. *Int J Cardiol* **31,** 199–204 (1991).
75. Kovanen, P., Mänttäri, M., Palosuo, T., Manninen, V. & Aho, K. Prediction of myocardial infarction in dyslipidemic men by elevated levels of immunoglobulin classes A, E, and G, but not M. *Arch. Intern. Med.* **158,** 1434–1439 (1998).
76. Erdogan, O., Gul, C., Altun, a. & Ozbay, G. Increased Immunoglobulin E Response in Acute Coronary Syndromes. *Angiology* **54,** 73–79 (2003).
77. Foks, A. C. *et al.* Interruption of the OX40-OX40 ligand pathway in LDL receptor-deficient mice causes regression of atherosclerosis. *J. Immunol.* **191,** 4573–4580 (2013).
78. Wang, J. *et al.* IgE stimulates human and mouse arterial cell apoptosis and cytokine expression and promotes atherogenesis in Apoe-/- mice. *J. Clin. Invest.* **121,** 3564–3577 (2011).
79. Varadaradjalou, S. *et al.* Toll-like receptor 2 (TLR2) and TLR4 differentially activate human mast cells. **2,** 899–906 (2003).
80. Lappalainen, J., Lindstedt, K. A., Oksjoki, R. & Kovanen, P. T. OxLDL-IgG immune complexes induce expression and secretion of proatherogenic cytokines by cultured human mast cells. *Atherosclerosis* **214,** 357–363 (2011).
81. Lopes-Virella, M. F. & Virella, G. Pathogenic Role of Modified LDL Antibodies and Immune Complexes in Atherosclerosis. *J. Atheroscler. Thromb.* **20,** 743–754 (2013).

82. Meng, Z. *et al.* Oxidized low-density lipoprotein induces inflammatory responses in cultured human mast cells via Toll-like receptor 4. *Cell. Physiol. Biochem.* **31,** 842–53 (2013).

83. Bot, M. *et al.* Lysophosphatidic acid triggers mast cell-driven atherosclerotic plaque destabilization by increasing vascular inflammation. *J. Lipid Res.* **54,** 1265–1274 (2013).

84. Oksjoki, R. *et al.* Receptors for the anaphylatoxins C3a and C5a are expressed in human atherosclerotic coronary plaques. *Atherosclerosis* **195,** 90–99 (2007).

85. de Vries, M. R. *et al.* Complement factor C5a as mast cell activator mediates vascular remodelling in vein graft disease. *Cardiovasc. Res.* **97,** 311–20 (2013).

86. Erdei, A., Andrasfalvy, M., Peterfy, H., Toth, G. & Pecht, I. Regulation of mast cell activation by complement-derived peptides. *Immunol. Lett.* **92,** 39–42 (2004).

87. Lerman, A. *et al.* Endothelin in coronary endothelial dysfunction and early atherosclerosis in humans. *Circulation* **92,** 2426–31 (1995).

88. Murray, D. B., Gardner, J. D., Brower, G. L. & Janicki, J. S. Endothelin-1 mediates cardiac mast cell degranulation, matrix metalloproteinase activation, and myocardial remodeling in rats. *Am. J. Physiol. Heart Circ. Physiol.* **287,** H2295--9 (2004).

89. Metsärinne, K. P. *et al.* Activated mast cells increase the level of endothelin-1 mRNA in cocultured endothelial cells and degrade the secreted peptide. *Arterioscler. Thromb. Vasc. Biol.* **22,** 268–273 (2002).

90. Swindle, E. J. & Metcalfe, D. D. The role of reactive oxygen species and nitric oxide in mast cell-dependent inflammatory processes. *Immunological Reviews* **217,** 186–205 (2007).

91. Laine, P., Naukkarinen, A., Heikkila, L., Penttila, A. & Kovanen, P. T. Adventitial Mast Cells Connect With Sensory Nerve Fibers in Atherosclerotic Coronary Arteries. *Circulation* **101,** 1665–1669 (2000).

92. Hartung, H. P. & Toyka, K. V. Substance P, the immune system and inflammation. *Int. Rev. Immunol.* **4,** 229–49 (1989).

93. Helke, C. J., Charlton, C. G. & Keeler, J. R. Bulbospinal substance P and sympathetic regulation of the cardiovascular system: A review. *Peptides* **6,** 69–74 (1985).

94. Cooke, H. J., Fox, P., Alferes, L., Fox, C. C. & Wolfe, S. A. Presence of NK1 receptors on a mucosal-like mast cell line, RBL-2H3 cells. *Can. J. Physiol. Pharmacol.* **76,** 188–193 (1998).

95. Lagraauw, H. M. *et al.* Vascular neuropeptide Y contributes to atherosclerotic plaque progression and perivascular mast cell activation. *Atherosclerosis* **235,** 196–203 (2014).

96. Ma, H. & Kovanen, P. T. Inhibition of mast cell-dependent conversion of cultured macrophages into foam cells with antiallergic drugs. *Arterioscler. Thromb. Vasc. Biol.* **20,** E134–E142 (2000).

97. Smith, D. D. *et al.* Mast cell deficiency attenuates progression of atherosclerosis and hepatic steatosis in apolipoprotein E-null mice. *AJP Hear. Circ. Physiol.* **302,** H2612–H2621 (2012).

98. Wang, J. *et al.* Pharmaceutical stabilization of mast cells attenuates experimental atherogenesis in low-density lipoprotein receptor-deficient mice. *Atherosclerosis* **229,** 304–309 (2013).

99. Saarinen, J., Kalkkinen, N., Welgus, H. G. & Kovanen, P. T. Activation of human interstitial procollagenase through direct cleavage of the Leu83-Thr84 bond by mast cell chymase. *J. Biol. Chem.* **269,** 18134–18140 (1994).

100. Tchougounova, E. *et al.* A key role for mast cell chymase in the activation of pro-matrix metalloprotease-9 and pro-matrix metalloprotease-2. *J. Biol. Chem.* **280,** 9291–9296 (2005).

101. Okumura, K. *et al.* Human chymase degrades human fibronectin [1]. *Clinica Chimica Acta* **347,** 223–225 (2004).

102. Leskinen, M. J., Lindstedt, K. A., Wang, Y. & Kovanen, P. T. Mast cell chymase induces smooth muscle cell apoptosis by a mechanism involving fibronectin degradation and disruption of focal adhesions. *Arterioscler. Thromb. Vasc. Biol.* **23,** 238–243 (2003).

103. Leskinen, M. J. *et al.* Mast cell chymase induces smooth muscle cell apoptosis by disrupting NF-kappaB-mediated survival signaling. *Exp. Cell Res.* **312,** 1289–1298 (2006).

104. Den Dekker, W. K. *et al.* Mast cells induce vascular smooth muscle cell apoptosis via a toll-like receptor 4 activation pathway. *Arterioscler. Thromb. Vasc. Biol.* **32,** 1960–1969 (2012).

105. Wang, Y., Shiota, N., Leskinen, M. J., Lindstedt, K. A. & Kovanen, P. T. Mast cell chymase inhibits smooth muscle cell growth and collagen expression in vitro: transforming growth factor-beta1-dependent and -independent effects. *Arterioscler. Thromb. Vasc. Biol.* **21,** 1928–1933 (2001).

106. Mayranpaa, M. I., Heikkila, H. M., Lindstedt, K. A., Walls, A. F. & Kovanen, P. T. Desquamation of human coronary artery endothelium by human mast cell proteases: implications for plaque erosion. *Coron. Artery Dis.* **17,** 611–621 (2006).

107. Heikkilä, H. M. *et al.* Activated mast cells induce endothelial cell apoptosis by a combined action of chymase and tumor necrosis factor-α. *Arterioscler. Thromb. Vasc. Biol.* **28,** (2008).

108. Taipale, J., Lohi, J., Saarinen, J., Kovanen, P. T. & Keski-Oja, J. Human mast cell chymase and leukocyte elastase release latent transforming growth factor-beta 1 from the extracellular matrix of cultured human epithelial and endothelial cells. *J. Biol. Chem.* **270,** 4689–4696 (1995).

109. Pejler, G., Rönnberg, E., Waern, I. & Wernersson, S. Mast cell proteases: Multifaceted regulators of inflammatory disease. *Blood* **115,** 4981–4990 (2010).

110. Lee, M., Calabresi, L., Chiesa, G., Franceschini, G. & Kovanen, P. T. Mast cell chymase degrades apoe and apoA-II in apoA-I-knockout mouse plasma and reduces its ability to promote cellular cholesterol efflux. *Arterioscler. Thromb. Vasc. Biol.* **22,** 1475–1481 (2002).

111. Lee-Rueckert, M. *et al.* Mast cell activation in vivo impairs the macrophage reverse cholesterol transport pathway in the mouse. *Arterioscler. Thromb. Vasc. Biol.* **31,** 520–527 (2011).

112. Lee, M., Lindstedt, L. K. & Kovanen, P. T. Mast cell-mediated inhibition of reverse cholesterol transport. *Arterioscler. Thromb.* **12,** 1329–1335 (1992).

113. Urata, H. Pathological involvement of chymase-dependent angiotensin II formation in the development of cardiovascular disease. *J. Renin. Angiotensin. Aldosterone. Syst.* **1,** S35-7 (2000).

114. Dell'Italia, L. J. & Husain, A. Dissecting the role of chymase in angiotensin II formation and heart and blood vessel diseases. *Curr. Opin. Cardiol.* **17,** 374–9 (2002).

115. Sasaki, S. & Inoguchi, T. The role of oxidative stress in the pathogenesis of diabetic vascular complications. *Diabetes Metab. J.* **36,** 255–61 (2012).

116. Ihara, M. *et al.* Increased chymase-dependent angiotensin II formation in human atherosclerotic aorta. *Hypertension* **33,** 1399–1405 (1999).

117. Guo, T., Chen, W. Q., Zhang, C., Zhao, Y. X. & Zhang, Y. Chymase activity is closely related with plaque vulnerability in a hamster model of atherosclerosis. *Atherosclerosis* **207,** 59–67 (2009).

118. Li, Y., Song, Y. H., Mohler, J. & Delafontaine, P. ANG II induces apoptosis of human vascular smooth muscle via extrinsic pathway involving inhibition of Akt phosphorylation and increased FasL expression. *Am J Physiol Hear. Circ Physiol* **290,** H2116-23 (2006).

119. Nabah, Y. N. A. *et al.* Angiotensin II induces neutrophil accumulation in vivo through generation and release of CXC chemokines. *Circulation* **110,** 3581–3586 (2004).

120. Bot, I. *et al.* Mast cell chymase inhibition reduces atherosclerotic plaque progression and improves plaque stability in ApoE-/- mice. *Cardiovasc. Res.* **89,** 244–252 (2011).

121. Ramalho, L. S. *et al.* Role of mast cell chymase and tryptase in the progression of atherosclerosis: study in 44 autopsied cases. *Ann. Diagn. Pathol.* **17,** 28–31 (2013).

122. Johnson, J. L., Jackson, C. L., Angelini, G. D. & George, S. J. Activation of matrix-degrading metalloproteinases by mast cell proteases in atherosclerotic plaques. *Arterioscler. Thromb. Vasc. Biol.* **18,** 1707–1715 (1998).

123. Yeong, P., Ning, Y., Xu, Y., Li, X. & Yin, L. Tryptase promotes human monocyte-derived macrophage foam cell formation by suppressing LXRalpha activation. *Biochim. Biophys. Acta* **1801,** 567–76 (2010).

124. Kinoshita, M., Okada, M., Kara, M., Furukawa, Y. & Matsumori, A. Mast cell tryptase in mast cell granules enhances MCP-1 and interleukin-8 production in human endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* **25,** 1858–1863 (2005).

125. Compton, S. J., Cairns, J. A., Holgate, S. T. & Walls, A. F. Human mast cell tryptase stimulates the release of an IL-8-dependent neutrophil chemotactic activity from human umbilical vein endothelial cells (HUVEC). *Clin. Exp. Immunol.* **121,** 31–36 (2000).

126. Lee, M. *et al.* Mast cell tryptase degrades HDL and blocks its function as an acceptor of cellular cholesterol. *Arterioscler. Thromb. Vasc. Biol.* **22,** 2086–2091 (2002).

127. Pang, L., Nie, M., Corbett, L., Sutcliffe, A. & Knox, A. J. Mast cell beta-tryptase selectively cleaves eotaxin and RANTES and abrogates their eosinophil chemotactic activities. *J. Immunol.* **176,** 3788–95 (2006).

128. Zhi, X. *et al.* Tryptase promotes atherosclerotic plaque haemorrhage in ApoE-/- mice. *PLoS One* **8,** e60960 (2013).

129. Inoue, T. *et al.* Vascular inflammation and repair: Implications for re-endothelialization, restenosis, and stent thrombosis. *JACC Cardiovasc. Interv.* **4,** 1057–1066 (2011).

130. Weintraub, W. S. The Pathophysiology and Burden of Restenosis. *Am. J. Cardiol.* **100,** (2007).

131. Shiota, N. *et al.* Tranilast suppresses vascular chymase expression and neointima formation in balloon-injured dog carotid artery. *Circulation* **99,** 1084–1090 (1999).

132. Jin, D. *et al.* Roles of chymase in stenosis occurring after polytetrafluoroethylene graft implantations. *Life Sci.* **81,** 1291–1300 (2007).

133. Miyazaki, M., Shiota, N., Sakonjo, H. & Takai, S. Angiotensin II Type 1 Receptor Antagonist, TCV-116, Prevents Neointima Formation in Injured Arteries in the Dog. *Jpn J Pharmacol* 455–460 (1999).

134. Tennant, G. M., Wadsworth, R. M. & Kennedy, S. PAR-2 mediates increased inflammatory cell adhesion and neointima formation following vascular injury in the mouse. *Atherosclerosis* **198,** 57–64 (2008).

135. Bell, L. & Madri, J. A. Effect of platelet factors on migration of cultured bovine aortic endothelial and smooth muscle cells. *Circ. Res.* **65,** 1057–65 (1989).

136. Fang, Y. I. *et al.* Marked increase in the histamine content of neointima after stent implantation of pig coronary artery and growth-promoting effects of histamine in cultured smooth muscle cells. *Life Sci.* **77,** 241–251 (2005).

137. Sasaguri, Y. & Tanimoto, A. Role of macrophage-derived histamine in atherosclerosis--chronic participation in the inflammatory response --. *J. Atheroscler. Thromb.* **11,** 122–30 (2004).

138. Mannaioni, P. F. *et al.* Storage and release of histamine in human platelets. *Inflamm. Res.* **44 Suppl 1,** S16-7 (1995).

139. Wessely, R. New drug-eluting stent concepts. *Nat. Rev. Cardiol.* **7,** 194–203 (2010).

140. Oh, B. & Lee, C. H. Nanofiber-coated drug eluting stent for the stabilization of mast cells.

Pharm. Res. **31,** 2463–2478 (2014).

141. Kounis, N. G. Coronary hypersensitivity disorder: The kounis syndrome. *Clinical Therapeutics* **35,** 563–571 (2013).
142. Kounis, N. G., Hahalis, G. & Theoharides, T. C. Coronary stents, hypersensitivity reactions, and the Kounis syndrome. *J. Interv. Cardiol.* **20,** 314–323 (2007).
143. Fuster, V., Badimon, L., Badimon, J. J. & H.Chesebro, J. The pathogenesis of coronary artery disease and the acute coronary syndromes. *N. Engl. J. Med.* (1992).
144. Jin, D. *et al.* Possible roles of cardiac chymase after myocardial infarction in hamster hearts. *Jpn. J. Pharmacol.* **86,** 203–14 (2001).
145. Jin, D. *et al.* Impact of chymase inhibitor on cardiac function and survival after myocardial infarction. *Cardiovasc. Res.* **60,** 413–420 (2003).
146. Wei, C. C. *et al.* Mast cell chymase limits the cardiac efficacy of Ang I-converting enzyme inhibitor therapy in rodents. *J. Clin. Invest.* **120,** 1229–1239 (2010).
147. Kanemitsu, H. *et al.* Chymase inhibition prevents cardiac fibrosis and dysfunction after myocardial infarction in rats. *Hypertens Res* **29,** 57–64 (2006).
148. Oyamada, S., Bianchi, C., Takai, S., Chu, L. M. & Sellke, F. W. Chymase inhibition reduces infarction and matrix metalloproteinase-9 activation and attenuates inflammation and fibrosis after acute myocardial ischemia/reperfusion. *J. Pharmacol. Exp. Ther.* **339,** 143–151 (2011).
149. Nistri, S. *et al.* Relaxin induces mast cell inhibition and reduces ventricular arrhythmias in a swine model of acute myocardial infarction. *Pharmacol Res* **57,** 43–48 (2008).
150. Matsumori, A. *et al.* Plasma levels of the monocyte chemotactic and activating factor/monocyte chemoattractant protein-1 are elevated in patients with acute myocardial infarction. *J Mol Cell Cardiol* **29,** 419–423 (1997).
151. Somasundaram, P. *et al.* Mast cell tryptase may modulate endothelial cell phenotype in healing myocardial infarcts. *J. Pathol.* **205,** 102–111 (2005).
152. Kwon, J. S. *et al.* The novel role of mast cells in the microenvironment of acute myocardial infarction. *J. Mol. Cell. Cardiol.* **50,** 814–825 (2011).
153. De Jesus, N. M. *et al.* Atherosclerosis exacerbates arrhythmia following myocardial infarction: Role of myocardial inflammation. *Heart Rhythm* **12,** 169–178 (2015).
154. Vukmir, R. B. Cardiac arrhythmia diagnosis. *Am. J. Emerg. Med.* **13,** 204–210 (1995).
155. S., A. E. *et al.* Carotid intima-media thickness is associated with incidence of hospitalized atrial fibrillation. *Atherosclerosis* **233,** 673–678 (2014).
156. STEVENSON, W. G. Ventricular Tachycardia After Myocardial Infarction: From Arrhythmia Surgery to Catheter Ablation. *J. Cardiovasc. Electrophysiol.* **6,** 942–950 (1995).
157. Wolff, a. a. & Levi, R. Histamine and cardiac arrhythmias. *Circ. Res.* **58,** 1–16 (1986).
158. Dai, S. Ventricular histamine concentrations and arrhythmias during acute myocardial ischaemia in rats. *Agents Actions* **21,** 66–71 (1987).
159. Wolff, A. A. & Levi, R. Ventricular arrhythmias parallel cardiac histamine efflux after coronary artery occlusion in the dog. *Agents Actions* **25,** 296–306 (1988).
160. Skovgaard, N., Moller, K., Gesser, H. & Wang, T. Histamine induces postprandial tachycardia through a direct effect on cardiac H2-receptors in pythons. *AJP Regul. Integr. Comp. Physiol.* **296,** R774–R785 (2008).
161. Seyedi, N. *et al.* Histamine H3-receptor-induced attenuation of norepinephrine exocytosis: a decreased protein kinase a activity mediates a reduction in intracellular calcium. *J. Pharmacol. Exp. Ther.* **312,** 272–80 (2005).
162. He, G. *et al.* Arrhythmogenic effect of sympathetic histamine in mouse hearts subjected to acute ischemia. *Mol. Med.* **18,** 1–9 (2012).
163. Jin, D. *et al.* An antiarrhythmic effect of a chymase inhibitor after myocardial infarction. *J.*

Pharmacol. Exp. Ther. **309,** 490–7 (2004).

164. Morrey, C. *et al.* Interaction between sensory C-fibers and cardiac mast cells in ischemia/reperfusion: activation of a local renin-angiotensin system culminating in severe arrhythmic dysfunction. *J. Pharmacol. Exp. Ther.* **335,** 76–84 (2010).
165. Koda, K. *et al.* Aldehyde dehydrogenase activation prevents reperfusion arrhythmias by inhibiting local renin release from cardiac mast cells. *Circulation* **122,** 771–781 (2010).
166. Liao, C. H. *et al.* Cardiac mast cells cause atrial fibrillation through PDGF-A - Mediated fibrosis in pressure-overloaded mouse hearts. *J. Clin. Invest.* **120,** 242–253 (2010).
167. Davis, F. M., Rateri, D. L. & Daugherty, A. Mechanisms of aortic aneurysm formation: translating preclinical studies into clinical therapies. *Heart* **100,** 1498–1505 (2014).
168. Reimerink, J. J., Van Der Laan, M. J., Koelemay, M. J., Balm, R. & Legemate, D. A. Systematic review and meta-analysis of population-based mortality from ruptured abdominal aortic aneurysm. *British Journal of Surgery* **100,** 1405–1413 (2013).
169. Faleiro, L. C., Machado, C. R., Gripp, A., Resende, R. A. & Rodrigues, P. A. Cerebral vasospasm: presence of mast cells in human cerebral arteries after aneurysm rupture. *J. Neurosurg.* **54,** 733–735 (1981).
170. Ishibashi, R., Aoki, T., Nishimura, M., Hashimoto, N. & Miyamoto, S. Contribution of mast cells to cerebral aneurysm formation. *Curr Neurovasc Res* **7,** 113–124 (2010).
171. Hasan, D., Chalouhi, N., Jabbour, P. & Hashimoto, T. Macrophage imbalance (M1 vs. M2) and upregulation of mast cells in wall of ruptured human cerebral aneurysms: preliminary results. *J. Neuroinflammation* **9,** 708 (2012).
172. Ollikainen, E. *et al.* Mast Cells, Neovascularization, and Microhemorrhages are Associated With Saccular Intracranial Artery Aneurysm Wall Remodeling. *J. Neuropathol. Exp. Neurol.* **73,** 855–64 (2014).
173. Zhang, J. *et al.* Chemokine (C-C motif) receptor 2 mediates mast cell migration to abdominal aortic aneurysm lesions in mice. *Cardiovasc. Res.* **96,** 543–51 (2012).
174. Sun, J. *et al.* Mast cells modulate the pathogenesis of elastase-induced abdominal aortic aneurysms in mice. *J. Clin. Invest.* **117,** 3359–3368 (2007).
175. Tsunemi, K. *et al.* A specific chymase inhibitor, 2-(5-formylamino-6-oxo-2-phenyl-1,6-dihydropyrimidine-1-yl)-N-[[3,4-dioxo-1-pheny l-7-(2-pyridyloxy)]-2-heptyl]acetamide (NK3201), suppresses development of abdominal aortic aneurysm in hamsters. *J. Pharmacol. Exp. Ther.* **309,** 879–883 (2004).
176. Sun, J. *et al.* Critical role of mast cell chymase in mouse abdominal aortic aneurysm formation. *Circulation* **120,** 973 (2009).
177. Zhang, J. *et al.* Mast cell tryptase deficiency attenuates mouse abdominal aortic aneurysm formation. *Circ. Res.* **108,** 1316–1327 (2011).
178. Mäyränpää, M. I. *et al.* Mast cells associate with neovessels in the media and adventitia of abdominal aortic aneurysms. *J. Vasc. Surg.* **50,** 388–395 (2009).
179. Tsuruda, T. *et al.* Adventitial mast cells contribute to pathogenesis in the progression of abdominal aortic aneurysm. *Circ. Res.* **102,** 1368–1377 (2008).
180. Wang, J. *et al.* IgE actions on $CD4^+$ T cells, mast cells, and macrophages participate in the pathogenesis of experimental abdominal aortic aneurysms. *EMBO Mol. Med.* **6,** (2014).
181. Tsuruda, T. *et al.* Adrenomedullin in mast cells of abdominal aortic aneurysm. *Cardiovasc. Res.* **70,** 158–164 (2006).
182. Holmes, D. R. *et al.* Results of prevention of REStenosis with tranilast and its outcomes (PRESTO) trial. *Circulation* **106,** 1243–1250 (2002).
183. Kaur, R. *et al.* A phenotypic screening approach in cord blood-derived mast cells to identify anti-inflammatory compounds. *J. Biomol. Screen.* **18,** 1223–33 (2013).
184. Sibilano, R., Frossi, B. & Pucillo, C. E. Mast cell activation: a complex interplay of positive

and negative signaling pathways. *Eur. J. Immunol.* **44,** 2558–2566 (2014).

185. Borriello, F. *et al.* Immunopharmacological modulation of mast cells. *Current Opinion in Pharmacology* **17,** 45–57 (2014).
186. Zhao, W., Oskeritzian, C. A., Pozez, A. L. & Schwartz, L. B. Cytokine production by skin-derived mast cells: endogenous proteases are responsible for degradation of cytokines. *J. Immunol.* **175,** 2635–42 (2005).
187. Lu, L.-F. *et al.* Mast cells are essential intermediaries in regulatory T-cell tolerance. *Nature* **442,** 997–1002 (2006).
188. Shefler, I., Salamon, P., Hershko, A. Y. & Mekori, Y. a. Mast cells as sources and targets of membrane vesicles. *Curr. Pharm. Des.* **17,** 3797–804 (2011).
189. Dimitris, S., Hany, G. B., Michèle, R. & Salah, M. Immunoregulatory properties of mast cell-derived exosomes. in *Molecular Immunology* **38,** 1359–1362 (2002).
190. Oldford, S. A. & Marshall, J. S. Mast cells as targets for immunotherapy of solid tumors. *Molecular Immunology* **63,** 113–124 (2014).

Chapter 3

Inhibition of lysophosphatidic acid receptors 1 and 3 attenuates atherosclerosis development in LDL-receptor deficient mice

Sci Rep. 2016; 6:37585

[1]Eva Kritikou
[1]Gijs H.M. van Puijvelde
[1]Thomas van der Heijden
[1]Peter J. van Santbrink
[1]Maarten Swart
[1]Frank H. Schaftenaar
[1]Mara J. Kröner
[1]Johan Kuiper
[1]Ilze Bot

[1] Division of Biopharmaceutics, Leiden Academic Centre for Drug Research, Leiden University, Leiden, The Netherlands

Abstract

Lysophosphatidic acid (LPA) is a natural lysophospholipid present at high concentrations within lipid-rich atherosclerotic plaques. Upon local accumulation in the damaged vessels, LPA can act as a potent activator for various types of immune cells, through its specific membrane receptors $LPA_{1/3}$. LPA elicits chemotactic, pro-inflammatory and apoptotic effects that lead to atherosclerotic plaque progression. In this study we aimed to inhibit LPA signaling by means of $LPA_{1/3}$ antagonism using the small molecule Ki16425. We show that $LPA_{1/3}$ inhibition significantly impaired atherosclerosis progression. Treatment with Ki16425 also resulted in reduced CCL2 production and secretion, which led to less monocytes and neutrophil infiltration. Furthermore, we provide evidence that $LPA_{1/3}$ blockade enhanced the percentage of non-inflammatory, $Ly6C^{low}$ monocytes and $CD4^{+}CD25^{+}FoxP3^{+}$ T regulatory cells. Finally, we demonstrate that $LPA_{1/3}$ antagonism mildly reduced plasma LDL cholesterol levels. Therefore, pharmacological inhibition of $LPA_{1/3}$ receptors may prove a promising approach to diminish atherosclerosis development.

1. Introduction

Atherosclerosis is a lipid-driven chronic inflammatory syndrome, accountable for the majority of acute cardiac episodes and comprising, at present, a principal cause of death in Western societies[1]. The disorder initiates upon damage of the arterial endothelium, induced by high shear stress and excessive amounts of cholesterol in the form of low-density lipoproteins (LDL). LDL can accumulate within the subendothelial space, triggering the immune system to launch an inflammatory cascade[2,3]. Thereupon, circulating monocytes infiltrate and ingest modified LDL particles, differentiating into macrophage foam cells; the main components of an atherosclerotic plaque. An additional influx of pro-atherogenic innate cells, such as neutrophils[4] and mast cells[5], follows, along with the rise of specific adaptive immune responses through presentation of lipid antigens by antigen presenting cells[6]. This process results in the infiltration of various subtypes of T cells, the main one being $CD4^+$ T helper 1 (T_{H1}) cells[7-9]. Evidently, an uncontrollable increase in the atherosclerotic plaque size, or rupture of the plaque, may lead to life-threatening clinical events.

Lysophosphatidic acid (LPA) is a bioactive glycerophospholipid found elevated in the circulation of patients with acute coronary syndromes[8], and directly linked to hyperlipidemia[9,10]. The presence of LPA in the serum has been mainly associated with platelet activation[11], but has also been described inside human atherosclerotic specimens[12], as well as in the plaques of $LDLr^{-/-}$ mice, with an increasing concentration rate upon disease progression[13]. Intraplaque LPA is enzymatically formed *in situ,* upon mild modification of the LDL molecule[14] and elicits its effects *via* 9 different G-protein coupled receptors ($LPA_{1\text{-}6,}$ GPR87, P2Y10 and GPR35) which have been classified into different subcategories based on their structural diversity and ligand specificity[15,16]. Of these receptors, LPA_1, LPA_2 and LPA_3 are structurally similar and belong to the Endothelial Differentiation Gene (EDG) family of proteins[17], with their general mode of action leading to gene regulation, chemokine secretion and cell survival[18]. Interestingly, LPA can activate mast cells[19,20] and neutrophils[21], as well as influence the migration of $CD4^+$ T helper cells[22]; all key cell types that are directly implicated in atherosclerosis. The impact of LPA on atherosclerosis has been thoroughly studied in the past, and reviewed in detail by Schober & Siess[23], with its role extending from increased infiltration and activation of monocytes to enhanced foam cell formation and elevated levels of endothelial permeability. Specifically, the chemotactic effects induced through LPA are linked to upregulation of inflammatory chemokines and adhesion molecules such as CCL2[24], CXCL1[25] and I-CAM 1[26]. The pro-atherosclerotic effects of LPA have been mainly tied to LPA_1 and $LPA_{3,}$ which were reported to increase atherosclerosis development in $apoE^{-/-}$ mice[27]. Receptors LPA_1 and LPA_3 are widely expressed by immune cells[28,29], as well as endothelial[30] and vascular smooth muscle cells[31], with LPA_3 activation being

involved in cell migration[32], while LPA_1 shows both migratory[33] and apoptotic effects[34].

In this study, we aimed to examine the development of atherosclerosis upon pharmacological blockade of receptors LPA_1 and LPA_3 ($LPA_{1/3}$), using the synthetic antagonistic compound Ki16425[35].

2. Materials & Methods

2.1 Animals

$LDLr^{-/-}$ mice were initially obtained from the Jackson Laboratories and bred at the Leiden University animal facility, with water and food supply *ad libitum*. All animal work was approved by the Leiden University Animal Ethics committee and performed according to the guidelines established by the Dutch government and European Union.

2.2 Atherosclerosis

Atherosclerosis was induced in male $LDLr^{-/-}$ mice (10-11 weeks old) by feeding a Western type diet (WTD) (0.25% cholesterol, 15% cocoa butter; Special Diet Services, Essex, UK) for 2 weeks prior and throughout the course of the treatment. Subsequently, 12-15 mice per group were randomized based on age, weight and/or cholesterol levels and injected intraperitoneally 3 times a week with either a vehicle control or 5mg/kg of the $LPA_{1/3}$ antagonist, Ki16425; the injections were performed for 6 weeks. All mice were monitored for body weight changes every week throughout the treatment period; at the experimental endpoint, weight was additionally determined in organs such as the spleen and liver.

2.3 Immunohistochemistry

The mice were anaesthetized with a mix of ketamine (100mg/ml), sedazine (25mg/ml) and atropine (0.5mg/ml) and perfused with PBS through heart puncture in the ventricles. The hearts were dissected below the atria and sectioned perpendicularly to the axis of the aorta, starting within the heart and in the direction towards the aortic arch. Upon aortic root identification by the appearance of the aortic valve leaflets, 10μm sections were collected and mounted on gelatin-coated slides. Mean plaque area (in μm^2) was calculated for six sequential Oil-Red-O stained sections in distal direction, starting at the point where all three aortic valve leaflets first appeared. Plaque macrophages were stained using a MOMA-2 antibody at a 1:1000 concentration (rat IgG2b, Serotec Ltd.). For the MOMA-2 levels in the plaques, three subsequent sections displaying the highest plaque content per mouse were analyzed. Mast cells and neutrophils were visualized by staining with a Naphthol AS-D chloro-acetate esterase kit (Sigma Aldrich) and counted manually. A mast cell was considered resting when all granules were maintained inside the cell, while mast cells were assessed as activated when granules were deposited in the tissue surrounding the mast cell. Neutrophils were identified as round cells with a characteristic lobular nucleus and pink granular cytoplasm. T cell numbers were determined following a CD3 staining at a 1:150 concentration (clone S7P, ThermoScientific). All microscopic analyses were performed on a Leica DM-RE microscope and ORO, as well as MOMA-2, quantifications were carried out using Leica QWin software (Leica, Imaging Systems, UK) and through blinded independent analysis.

2.4 RT-PCR

Isolation of mRNA was performed on the liver and aortic arch of 8 mice/group, based on the guanidium isothiocyanate method[58]. Subsequently, the M-MuLV reverse transcriptase (RevertAid, Leon-Roth) was used for the reverse transcription. The quantitative analysis of specific gene expression was executed on a 7500 Fast-real time PCR system (Applied Biosystems, Foster City, CA) using SYBR Green Technology. The relative expression was determined based on two housekeeping genes; β-actin and ribosomal protein L27 (Rpl27). The complete primer list is included in the **Supplementary Table S1**.

2.5 Serum analysis

Throughout the study, tail blood was collected by a tail-vein cut; serum was obtained by centrifugation at 8.000 rpm for 10 minutes. Total cholesterol levels in the serum were measured at week 0, 3 and 6 of the treatment by an enzymatic colorimetric assay and in comparison to an internal Precipath control (standard serum provided by Roche Diagnostics). At the end of the experiment, cholesterol fraction separation for lipoprotein particle analysis was obtained after processing 30μL serum/mouse through a Superose 6 column (Smart System, Pharmacia). Subsequent measurement of cholesterol levels in the retrieved fractions was performed as described above. CCL2 levels in the serum of 13 mice/group were analyzed by ELISA (BD Biosciences) according to the manufacturer's protocol.

2.6 Flow cytometry

Blood was collected as described above; Red blood cells were lysed using an erythrocyte lysis buffer (0.1mM EDTA, 10mM $NaHCO_3$, 1mM NH_4Cl, pH=7.2). Subsequently, white blood cells were stained with the relevant antibodies for flow cytometry analysis (**Supplementary Table S2**). Spleen tissue was harvested from all mice and processed through a 70μm cell strainer to acquire single cell suspensions. Subsequently, cells underwent lysis once, for erythrocyte disposal, and were stained for flow cytometry. In approximation, 200.000 cells per sample were stained with antibodies against extracellular proteins at a concentration of 0.1 μg/sample for 30 minutes. For the detection of intracellular antibodies all cells were fixated, permeabilized using the transcription factor kit (Ebioscience) and stained at a concentration of 0.3 μg/sample. All flow cytometry experiments were executed on a FACS Canto II and data were analyzed using FlowJo software.

2.7 Statistics

All data are analyzed using the GraphPad Prism software and presented as mean±SEM. A 2-tailed Student's *t*-test was used to compare individual groups. Non-Gaussian distributed data were analyzed using a 2-tailed Mann-Whitney *U* test. For the analysis of two or more variables a two-way ANOVA was used with the Bonferroni post-test for multiple comparisons. The probability (alpha) for all tests was set to 0.05 with values lower than this considered significant ($P<0.05$).

3. Results

3.1 $LPA_{1/3}$ inhibition reduces atherosclerotic plaque size

To assess the effect of $LPA_{1/3}$ inhibition on atherosclerosis, $LDLr^{-/-}$ mice were injected intraperitoneally with either Ki16425 (5mg/kg) or a vehicle-control for 6 weeks,

(3x/week). Plaque size quantification, using an Oil-Red-O staining showed that mice treated with the $LPA_{1/3}$ inhibitor had significantly smaller plaque size (-40%) compared to control mice (**Figure 1A**, Ki16425: $89*10^3 \pm 9*10^3$ µm² vs control: $147*10^3 \pm 21*10^3$ µm², P=0.023). In fact, plaque size was significantly lower in the treated group at each distance measured from the start of the three-valve area up to its end (**Figure 1B**). A MOMA-2 staining was performed to evaluate the intra-plaque macrophage levels. The absolute macrophage content of the Ki16425 treated group was significantly lower (-45%) compared to the control (**Figure 1C**, Ki16425: $36*10^3 \pm 8*10^3$ µm² vs control: $65*10^3 \pm 6*10^3$ µm², P=0.006), whereas the relative amount (% macrophage levels of the plaque) was not significantly different (**Figure 1D**, P=0.11). Furthermore, the aortic root area was analyzed for mast cell and neutrophil content, since both immune cell types express $LPA_{1/3}$ and are involved in atherosclerosis progression. No differences in the number or activation status of mast cells were detected between the two groups (**Figure 1E**, P=0.38 for mast cell # and Fig.1F, P=0.88 for activated mast cell #). However, a substantial reduction in the number of infiltrated neutrophils (-31%) upon $LPA_{1/3}$ blockade was observed (**Figure 1G**, Ki16425:8.5±0.7 neutrophils/µm² tissue vs. control: 12.4±0.9 neutrophils/µm² tissue, P=0.004).

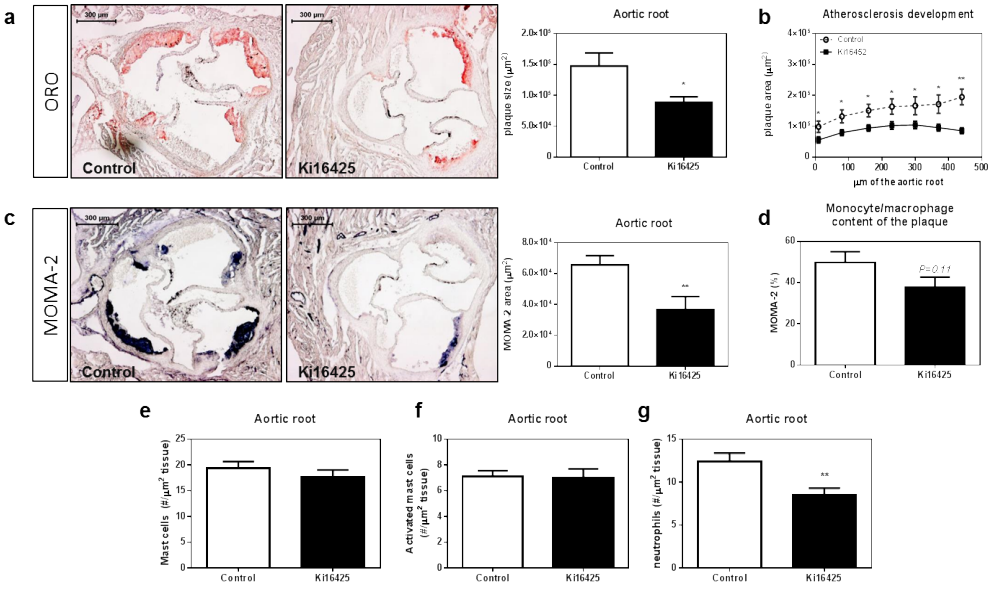


Figure 1: The $LPA_{1/3}$ antagonist, Ki16425, reduces atherosclerosis development. (a) Atherosclerotic plaque size in the aortic root of the heart was determined by an Oil-Red-O staining on 10µm sections; representative pictures are shown. Blockade of receptors $LPA_{1/3}$ resulted in a 40% reduction in atherosclerosis. **(b)** The Ki16425 treated mice had significantly lower atherosclerotic plaque development throughout the entire three valve area of the aortic root. **(c)** Macrophage expression levels were measured using a MOMA-2 staining; $LPA_{1/3}$ antagonism led to 45% less macrophage accumulation within the aortic root of the hearts. **(d)** The relative amount of macrophages in the atherosclerotic plaques was not significantly affected by the Ki16425 treatment.

(e) Mast cell numbers (#) and **(f)** activation state, as well as **(g)** neutrophil numbers were manually quantified using a Naphthol AS-D chloro-acetate esterase staining; no difference was observed in the number or degranulation status of mast cells in the aortic root. Neutrophil numbers were found significantly reduced by 31% in the aortic root of the Ki16425 group as compared to the control. All values (n=12/grp) are depicted as mean±SEM (*P<0.05, **P<0.01).

3.2 Treatment with Ki16425 results in decreased serum total cholesterol levels

$LPA_{1/3}$ inhibition with Ki16425 did not alter body weight (**Figure 2A**) and similarly, analysis of the liver weight or spleen weight at the endpoint of the study presented no change for the treated versus the control group (**Supplementary Figure 1A,B**). Interestingly, total serum cholesterol levels in time upon $LPA_{1/3}$ inhibition, was significantly lower compared to the control group (**Figure 2B**, w3: -20%, P=0.012; w6: -16%, P=0.017). Further analysis of the serum lipoprotein content at the experimental endpoint showed a trend towards decreased LDL levels for the Ki16425 treated mice, as compared to the control (**Figure 2C**, P=0.06).

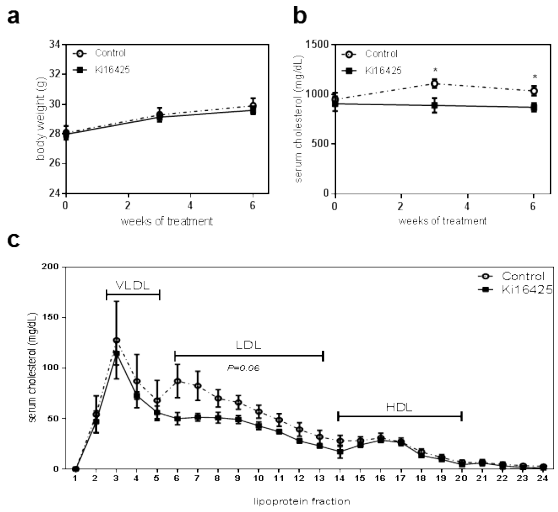


Figure 2: $LPA_{1/3}$ inhibition reduces total cholesterol content in the serum. (a) The animal body weight showed no significant differences throughout 6 weeks of treatment between the groups. **(b)** Total serum cholesterol remained at significantly lower levels in the Ki16425 group compared to the control. **(c)** The Ki16425 treated animals presented a trend towards reduced LDL levels (P=0.06). P-values are calculated by the fraction sum for each lipoprotein per group (n=5/grp). All values are depicted as mean±SEM (*P<0.05).

3.3 Pro-inflammatory CCL2 expression and secretion are reduced upon $LPA_{1/3}$ inhibition

Considering the lower macrophage and neutrophil content of the atherosclerotic plaque, we isolated mRNA from the aortic arch which was subsequently analyzed for the expression levels of different chemokines as well as endothelial adhesion molecules. The expression of ICAM-1, which is tightly linked to LPA[26], did not present any differences between the control and Ki16425 group (**Figure 3A**, P=0.70). The same was observed for

chemokine CXCL1 (**Figure 3B**, P=0.52). The pro-inflammatory chemokine CCL2 showed a trend towards reduction in the aortic arch of the Ki16425 treated mice (**Figure 3C**, P=0.067). Similarly, liver mRNA analysis displayed substantially lower CCL2 expression upon $LPA_{1/3}$ antagonism (**Figure 3D**, P=0.035). In addition, gene expression of the macrophage marker CD68 was significantly reduced in the liver of animals treated with Ki16425 (**Figure 3E**, P=0.037). To establish whether the reduction in CCL2 expression results in diminished protein levels, CCL2 chemokine secretion in the circulation was determined and found considerably lower (-65%) upon $LPA_{1/3}$ inhibition (**Figure 3F**, Ki16425: 58.9±6.4 pg/mL compared to 165.8±32.8 pg/mL, P=0.003).

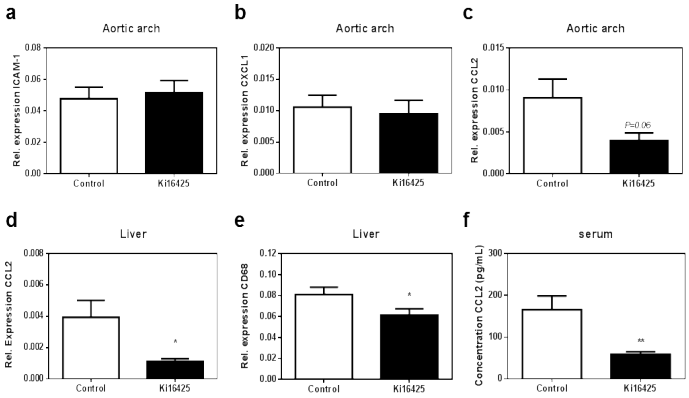


Figure 3: Treatment with Ki16425 decreases CCL2 chemokine expression and release. Gene profiling in the aortic arch displayed no difference between the two groups in **(a)** ICAM-1 or **(b)** CXCL1 chemokine expression levels. **(c)** CCL2 expression showed a slight reduction upon $LPA_{1/3}$ antagonism. **(d)** Liver CCL2 expression was significantly lower in the Ki16425 treated animals compared to the control. **(e)** Macrophage CD68 gene expression in the liver was reduced for the Ki16425 mice compared to the controls. **(f)** CCL2 chemokine secretion was decreased up to 65% in the circulation of the Ki16425 group, compared to the control. Gene expression (n=8/grp) is depicted as relative to two housekeeping genes (β-actin and Rpl27). Serum CCL2 concentration was measured using an ELISA assay (n=13/grp). All values were calculated as mean±SEM. (*P<0.05,**P<0.01).

3.4 $CCR2^+$ neutrophils and monocytes circulate at lower levels upon $LPA_{1/3}$ antagonism

Since the reduced CCL2 levels could lower the inflammatory cell infiltration, and therefore lower the plaque size, it was particularly intriguing to focus on immune cells that respond to this chemokine *via* its specific receptor, CCR2. For that reason, two additional groups of $LDLr^{-/-}$ mice were treated with either Ki16425 or vehicle-control for 6 weeks while on WTD. In the blood the overall percentage of circulating neutrophils, defined as $Ly6G^+CD11b^+/NK1.1^-$ cells, was not affected by the treatment (**Figure**

4A). However, the relative amount of circulating CCR2$^+$ neutrophils was significantly increased over time in the control group (**Figure 4B**, control: w0 compared to w2, P=0.031; w0 compared to w4, P=0.0001), but was found reduced in the treated animals after 4 weeks of $LPA_{1/3}$ inhibition (w4: Ki16425 compared to control, P=0.0060).

Furthermore, the monocyte population in the circulation, defined as Ly6C$^+$/CD11b$^+$Ly6G$^-$/NK1.1$^-$ cells was found to increase in the control group during 4 weeks of treatment (**Figure 4C**, control: w0 compared to w4, P=0.014). At week 4, the Ki16425 treated group showed a trend to reduced monocyte percentage when compared to the control (**Figure 4C**, w4: Ki16425 compared to control, P=0.066). Of this population, the CCR2$^+$ monocytes, which comprise the inflammatory monocyte subset[36] -alternatively defined as Ly6C^{high}- increased greatly in the control group over time (**Figure 4D**, control: w0 compared to w2, P=0.004; w0 compared to w4, P=0.0002), while the CCR2$^+$ monocytes of the treated group were only slightly elevated (Ki16425: w0 compared to w2, P=0.64; w0 compared to w4, P=0.054).

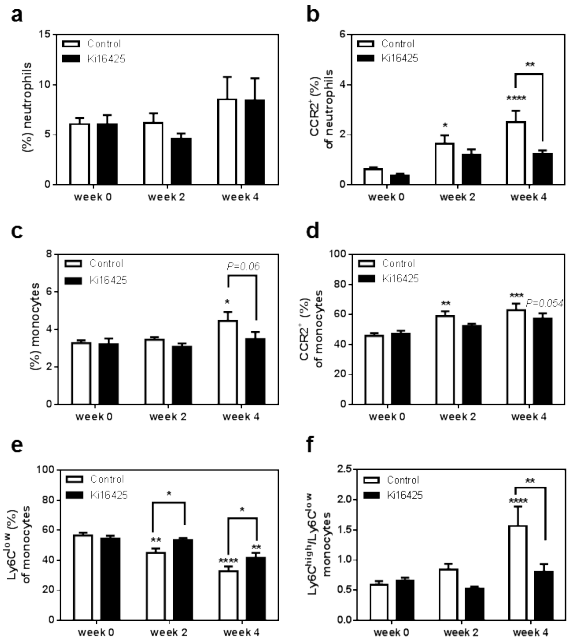


Figure 4: $LPA_{1/3}$ inhibition retains circulating CCR2$^+$ neutrophils and monocytes at low levels while increasing, Ly6C^{low} patrolling monocytes over time. (a) No difference was observed in the circulating neutrophil percentage between the two groups of animals. **(b)** CCR2$^+$ expressing neutrophils were reduced after 4 weeks of $LPA_{1/3}$ antagonism. **(c)** Circulating monocytes showed a slight reduction upon 4 weeks of Ki16425 treatment. **(d)** CCR2$^+$ monocytes remained at lower levels in the course of $LPA_{1/3}$ inhibition. **(e)** Non-inflammatory monocytes appeared significantly higher at 2 and 4 weeks of $LPA_{1/3}$ inhibition. **(f)** The ratio of inflammatory/non-inflammatory monocytes was increased in the control group compared to the treated. All values are calculated as mean±SEM. (n=12/grp, *P<0.05, **P<0.01, ***P<0.001, **** P<0.0001).

On the contrary, Ly6C^{low}-patrolling monocytes of the control mice showed a sharp decline over time (control: w0 compared to w2, P=0.0039; w0 compared to w4, P<0.0001), but upon Ki16425 treatment the decrease was only observed after 4 weeks and was less acute (Ki16425: w0 compared to w4, P=0.0025). Therefore, in relation to the control group, $LPA_{1/3}$ blocked animals presented significantly higher non-inflammatory circulating monocytes over time (**Figure 4E**, Ki16425 compared

to control: w2:P=0.048; w4:P=0.040). Therefore, $LPA_{1/3}$ inhibition retained the inflammatory versus non-inflammatory responses at lower levels as shown by the relative difference of inflammatory versus the non-inflammatory monocytes per group (**Figure 4F**, Ki16425 w4 compared to control w4: P=0.003).

3.5 $LPA_{1/3}$ inhibition increases circulating anti-inflammatory $CD4^+$ T regulatory cells, while decreasing T helper 1 cells

Flow cytometric analysis of the white blood cell population showed a significant reduction in the circulating $CD4^+$ T cell percentage after $LPA_{1/3}$ inhibition (**Figure 5A**, P=0.036). However, within the $CD4^+$ T cell population, anti-inflammatory $FoxP3^+/CD25^+/CD4^+$ T_{REG} percentage was found significantly increased (**Figure 5B**, Ki16425: 10.24 ± 0.71% versus control: 7.06 ± 0.41%, P=0.0013). Among the $CD4^+$ T_{REG} population, $FoxP3^+Helios^-/CD25^+$ cells, defined as inducible (i)T_{REG} and generated in secondary lymphoid organs[37,38], increased significantly in the Ki16425-treated group (**Figure 5C**, P=0.038). The percentage of pro-inflammatory $CD4^+$ T_{H1} cells in the blood was reduced upon $LPA_{1/3}$ inhibition (**Figure 5D**, P=0.011). Also in the spleen, $CD4^+$ T cells were reduced in the Ki16425 treated mice (Fig.5E, P<0.0001), with no difference observed however in the overall $FoxP3^+/CD25^+CD4^+$ T_{REG} population (**Figure 5F**, P=0.39). Nonetheless, $LPA_{1/3}$ inhibition resulted in a significant reduction in the inducible $CD4^+$ T_{REG} cells (**Figure 5G**, P=0.025). No significant differences in T_{H1} cells were observed in this compartment (**Figure 5H**, P=0.09).

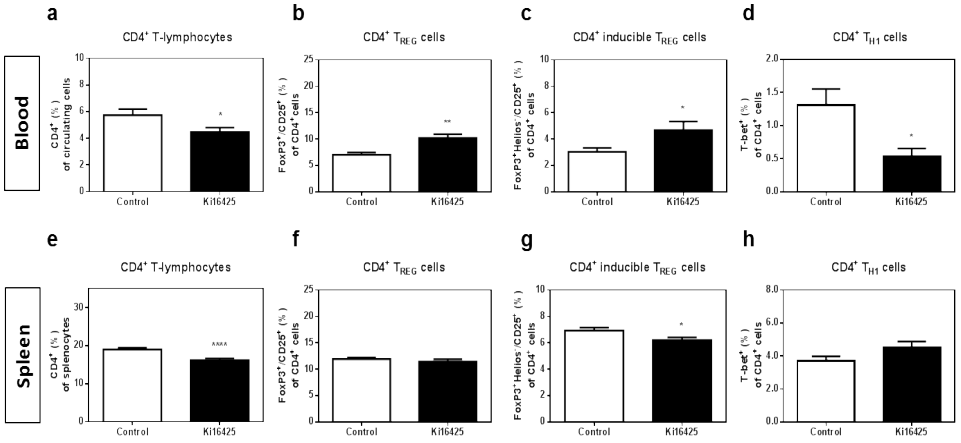


Figure 5: Systemic treatment with Ki16425 for 6 weeks reduces $CD4^+$ T cells in the blood and spleen, with a potency to increase anti-inflammatory T_{REG} cells. (a) Upon 6 weeks of treatment, $LPA_{1/3}$ antagonism reduced the percentage of $CD4^+$ T cells in the blood of $LPA_{1/3}$ blocked mice, as compared to the control. **(b)** Anti-inflammatory $CD4^+$ T_{REG} and **(c)** inducible $FoxP3^+Helios^-$ T_{REG} cells were detected at higher levels in contrast to **(d)** lower inflammatory T_{H1} cells in the blood of the Ki16425 treated mice. **(e)** In the spleen of Ki16425 treated animals $CD4^+$ T cells appeared significantly lower. **(f)** No difference was observed within

the overall $CD4^+$ T_{REG} cells. **(g)** The inducible T_{REG} cells were found substantially decreased. **(h)** No significant difference was detected in the $CD4^+$ T_{H1} population. All values are calculated as mean±SEM. (n=12/grp, *P<0.05,**P<0.01, ****P<0.0001).

In addition, no difference was observed within the $CD8^+$ population present in the blood or spleen of the two groups (**Supplementary Figures 2A,B**). Likewise, MHC-II^+CD11c^{high} dendritic cells (DCs) were not affected by the treatment (**Supplementary Figure 2C**).

3.6 The T cell content of the atherosclerotic plaque is not altered by $LPA_{1/3}$ inhibition

Considering that the systemic differences in the $CD4^+$ T cells upon $LPA_{1/3}$ blockade may directly influence the T cell content inside the atherosclerotic plaque, a $CD3^+$ immunohistochemical staining was performed in the aortic root. However, no significant differences were observed upon manual quantification of the $CD3^+$ cells in the three-valve area of both groups (**Figure 6**, P=0.43).

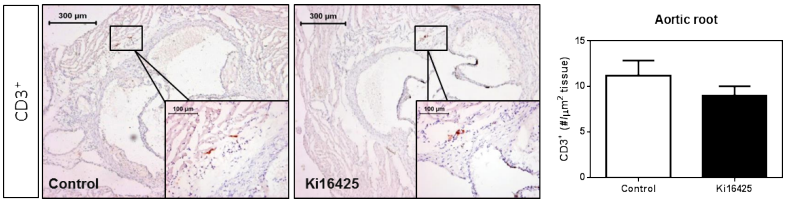


Figure 6: Total $CD3^+$ T cell numbers in the aortic root show no difference between the control and Ki16425-treated groups. $CD3^+$ cells in the aortic root of the hearts were determined upon manual quantification. No difference was observed in the $CD3^+$ expressing cells of the aortic root upon $LPA_{1/3}$ inhibition. All values (n=12/grp) are depicted as mean±SEM.

4. Discussion

In this study we show that pharmacological inhibition of $LPA_{1/3}$ receptors through Ki16425, reduced atherosclerotic plaque development by 40%. Specifically, we have found that $LPA_{1/3}$ antagonism significantly attenuated the macrophage and neutrophil content of the plaque. Furthermore, a mild downregulation of total serum cholesterol was detected, which may have contributed to the decreased atherosclerotic plaque size. We observed a systemic reduction in the expression and secretion of chemokine CCL2, which could have resulted in the detected $CCR2^+$ monocyte and neutrophil decline in the circulation. This decline in $CCR2^+$ cells in the blood seems consistent with the reduction in the absolute amount of macrophages and neutrophils inside the atherosclerotic plaques of the Ki16425 mice. Upon lower levels of circulating

CCL2, less CCR2^{+} monocytes and neutrophils are infiltrating the atherosclerotic site and subsequently there is a reduction in the absolute number of neutrophils and macrophages observed inside the plaque. As the atherosclerotic plaque at this specific timepoint consists primarily of macrophages, the absolute amount actually reflected the reduction in plaque size, whereas the relative macrophage content did not differ. This response is in line with previous evidence supporting that LPA elicits its effects through the release of CCL2 by its target cells[19]. Unlike reports that link the action of LPA to endothelial cell activation[12,26,39], in this investigation we did not observe any differences in the aortic ICAM-1 or CXCL1 expression levels. It is important to mention however that in a previously published study by Zhou et al., the atherosclerotic effects of LPA were mainly associated with endothelial activation through chemokine CXCL1[25] and not with the CCL2-CCR2 axis. Furthermore, in their study no effect was observed on cholesterol metabolism, whereas we detected a reduction in LDL cholesterol levels. Considering that the above experiments were performed in apoE$^{-/-}$ mice, it is possible that strain specific effects are responsible for the different mechanism observed[40]. In the past, numerous studies have examined the differences between the LDLr$^{-/-}$ and apoE$^{-/-}$ models. The leading distinction between the two strains is that LDLr$^{-/-}$ mice do not develop atherosclerosis unless placed on a high-cholesterol diet[41], whereas apoE$^{-/-}$ mice have basal plaque formation even on chow diet[42]. Thereupon, apoE$^{-/-}$ mice develop higher plasma cholesterol levels and more pronounced plaques upon WTD, while showing lower plaque T cell numbers[43]. In addition, apoE$^{-/-}$ mice, unlike the LDLr$^{-/-}$, exhibit impaired formation and efflux of HDL particles, an effect which is highly distinct from the human case[44]. With the above in mind, we considered LDLr$^{-/-}$ as a model that does not show atherosclerosis-related systemic effects prior to WTD. It is important to mention that the presence of apoE can affect monocyte formation and accumulation in the plaque[45], which could account for the main difference between the two models in regard to the role of $LPA_{1/3}$ receptors. In addition, apoE$^{-/-}$ and LDLr$^{-/-}$ mice have also proven to differ in the ABCA1-mediated cholesterol transportation pathway[46,47]. This may explain the reason why LDLr$^{-/-}$ mice show differences in cholesterol regulation upon $LPA_{1/3}$ inhibition, an effect which was absent in apoE$^{-/-}$ mice. Nevertheless, in both experimental sets $LPA_{1/3}$ inhibition substantially reduced plaque development to a similar extend despite the fact that the apoE$^{-/-}$ mice were treated with Ki16425 daily for 3 months while in our study LDLr$^{-/-}$ mice were injected 3x/week for a 6 week period.

Furthermore, in our study, the patrolling Ly6C^{low} population was increased already after two weeks of Ki16425 treatment, suggesting an additional anti-inflammatory effect. This anti-inflammatory subtype of mouse monocytes expressing the Ly6C protein at low levels, has been previously reported to play a crucial role in the control of atherosclerosis development since LDLr$^{-/-}$ mice that lack this subtype showed increased plaque formation[48]. Therefore, the anti-inflammatory effects of patrolling

monocytes detected upon $LPA_{1/3}$ antagonism may have contributed to the reduction in atherosclerosis development. Together, it seems that Ki16425 carries out its effects primarily by regulating the immune response, since also the $CCR2^+$ monocytes and neutrophils are circulating at lower levels compared to the control, already at the second week of treatment. This does not exclude the fact that cholesterol lowering mechanisms may have been partly responsible for the reduced disease progression.

In the past, LPA has also been reported to affect the proliferation of T lymphocytes[49]. Here we observe that upon $LPA_{1/3}$ antagonism, $CD4^+$ T cells are substantially diminished, which may have been due to a reduction in LPA induced mitogenesis[18]. Furthermore, LPA can promote the migration of $CD4^+$ T cells[33], but not of $CD8^+$ T cells[50]. This is in line with our observations on $CD4^+$ T cells being markedly reduced upon $LPA_{1/3}$ inhibition, while no effects were detectable on the $CD8^+$ T cell population in either the blood or spleen. Similarly, no differences were found in the dendritic cell population; however, the effects of LPA on DCs seem to depend on their state. For example, mouse DCs were previously reported to migrate towards LPA upon LPA_3 activation, yet for that to take place they had to be in an immature state[51]. As mentioned above, $CD4^+$ T cells can migrate in response to LPA, but this action was previously shown to depend on the expression of LPA_1 and LPA_2. Specifically, these two receptors are considered to have an inverse impact on T cell migration, with LPA_1 enhancing it while LPA_2 hinders it, depending though also on whether the cells are in a naive or activated state[50]. This fact further illustrates the complexity behind LPA signaling and how different receptor signaling pathways can have entirely opposing actions.

Notably, while $CD4^+$ T cell levels were reduced upon Ki16425 treatment, the anti-inflammatory T_{REG} cells were strongly enhanced. To our knowledge, there is thus far no evidence describing the relationship between $LPA_{1/3}$ inhibition and T_{REG} induction. The observed effects indicate that $LPA_{1/3}$ inhibition can skew the adaptive immune system towards an anti-inflammatory response as demonstrated by the overall lower $CD4^+$ levels, with the pro-inflammatory T_{H1} subset found relatively decreased in the blood while the athero-protective[52] $CD4^+$ T_{REG} cells were circulating at increased levels.

However, the differences on the $CD4^+$ population in the blood and spleen did not reflect the T cell numbers inside the plaques of the treated mice. This suggests that the reduced atherosclerotic plaque size observed upon $LPA_{1/3}$ antagonism was not mediated through a difference in local T cell responses, but was rather elicited *via* a systemic anti-inflammatory effect of the Ki16425 treatment. Moreover, we found no difference on the mast cell counts in the atherosclerotic plaques of the treated mice, despite previous evidence that mast cells respond to LPA through $LPA_{1/3}$ receptors[53]. Recent studies have also implicated additional LPA receptors present on the mast cell surface, which may induce mast cell activation in an $LPA_{1/3}$ independent fashion[54]. Furthermore, mast cell

effects mediated by LPA could also depend on the state of atherosclerosis. For instance, our group has previously reported that mast cell activation through LPA leads to advanced plaque destabilization[19], while in this study we aimed our attention in early plaque development.

In conclusion, $LPA_{1/3}$ receptor inhibition through Ki16425 induced systemic anti-inflammatory responses *via* a reduction in CCL2-CCR2 signaling, an enhancement of anti-inflammatory innate, as well as adaptive, immune responses and a decrease in plasma cholesterol levels, collectively resulting in the reduction of the atherosclerotic burden. The anti-inflammatory immune reaction of Ki16425 may span even outside atherosclerosis and into other diseases, which are characterized by excessive inflammation. For instance, Ki16425 has been found to reduce inflammation in experimental models for obesity[55], rheumatoid arthritis[56], and lung fibrosis[57]. Thus, the importance of singularly targeting the $LPA_{1/3}$ receptors *via* pharmacological inhibition may grant anti-inflammatory effects without completely shutting down the immune response. At the same time it retains possible positive effects that LPA may evoke through its additional receptors in other physiological processes inside the body. Therefore, $LPA_{1/3}$ inhibition is an interesting therapy with multiple beneficial effects that can be employed in a broad spectrum of diseases, among which atherosclerosis.

Reference list:

1. Mozaffarian, D. *et al.* Heart Disease and Stroke Statistics-2016 Update: A Report From the American Heart Association. *Circulation* **133,** e38–e360 (2016).
2. Libby, P., Ridker, P. M. & Hansson, G. K. Progress and challenges in translating the biology of atherosclerosis. *Nature* **473,** 317–25 (2011).
3. Libby, P., Lichtman, A. H. & Hansson, G. K. Immune effector mechanisms implicated in atherosclerosis: from mice to humans. *Immunity* **38,** 1092–1104 (2013).
4. Drechsler, M., Megens, R. T. A., van Zandvoort, M., Weber, C. & Soehnlein, O. Hyperlipidemia-triggered neutrophilia promotes early atherosclerosis. *Circulation* **122,** 1837–1845 (2010).
5. Bot, I. *et al.* Mast cell chymase inhibition reduces atherosclerotic plaque progression and improves plaque stability in ApoE-/- mice. *Cardiovasc. Res.* **89,** 244–252 (2011).
6. Zernecke, A. Dendritic cells in atherosclerosis: evidence in mice and humans. *Arterioscler. Thromb. Vasc. Biol.* **35,** 763–770 (2015).
7. Frostegard, J. *et al.* Cytokine expression in advanced human atherosclerotic plaques: dominance of pro-inflammatory (Th1) and macrophage-stimulating cytokines. *Atherosclerosis* **145,** 33–43 (1999).
8. Dohi, T. *et al.* Increased circulating plasma lysophosphatidic acid in patients with acute coronary syndrome. *Clin. Chim. Acta.* **413,** 207–212 (2012).
9. Tokumura, A. *et al.* Increased formation of lysophosphatidic acids by lysophospholipase D in serum of hypercholesterolemic rabbits. *J. Lipid Res.* **43,** 307–315 (2002).
10. Yao, C.-S. *et al.* Patients with risk factors have higher plasma levels of lysophosphatidic acid: a promising surrogate marker for blood platelet activation. *Blood Coagul. Fibrinolysis* **25,** 322–325 (2014).
11. Eichholtz, T., Jalink, K., Fahrenfort, I. & Moolenaar, W. H. The bioactive phospholipid lysophosphatidic acid is released from activated platelets. *Biochem. J.* **291 (Pt 3,** 677–680 (1993).
12. Siess, W. *et al.* Lysophosphatidic acid mediates the rapid activation of platelets and endothelial cells by mildly oxidized low density lipoprotein and accumulates in human atherosclerotic lesions. *Proc. Natl. Acad. Sci. U. S. A.* **96,** 6931–6936 (1999).
13. Bot, M. *et al.* Atherosclerotic lesion progression changes lysophosphatidic acid homeostasis to favor its accumulation. *Am. J. Pathol.* **176,** 3073–3084 (2010).
14. Siess, W. Platelet interaction with bioactive lipids formed by mild oxidation of low-density lipoprotein. *Pathophysiol. Haemost. Thromb.* **35,** 292–304 (2006).
15. Chun, J., Hla, T., Lynch, K. R., Spiegel, S. & Moolenaar, W. H. International Union of Basic and Clinical Pharmacology. LXXVIII. Lysophospholipid receptor nomenclature. *Pharmacol. Rev.* **62,** 579–587 (2010).
16. Choi, J. W. *et al.* LPA receptors: subtypes and biological actions. *Annu. Rev. Pharmacol. Toxicol.* **50,** 157–186 (2010).
17. Fukushima, N. & Chun, J. The LPA receptors. *Prostaglandins Other Lipid Mediat.* **64,** 21–32 (2001).
18. Yung, Y. C., Stoddard, N. C. & Chun, J. LPA receptor signaling: pharmacology, physiology, and pathophysiology. *J. Lipid Res.* **55,** 1192–1214 (2014).
19. Bot, M. *et al.* Lysophosphatidic acid triggers mast cell-driven atherosclerotic plaque destabilization by increasing vascular inflammation. *J. Lipid Res.* **54,** 1265–1274 (2013).
20. Hashimoto, T., Ohata, H. & Honda, K. Lysophosphatidic acid (LPA) induces plasma exudation and histamine release in mice via LPA receptors. *J. Pharmacol. Sci.* **100,** 82–87 (2006).

21. Hashimoto, T., Yamashita, M., Ohata, H. & Momose, K. Lysophosphatidic acid enhances in vivo infiltration and activation of guinea pig eosinophils and neutrophils via a Rho/Rho-associated protein kinase-mediated pathway. *J. Pharmacol. Sci.* **91,** 8–14 (2003).
22. Wang, L., Knudsen, E., Jin, Y., Gessani, S. & Maghazachi, A. A. Lysophospholipids and chemokines activate distinct signal transduction pathways in T helper 1 and T helper 2 cells. *Cell. Signal.* **16,** 991–1000 (2004).
23. Schober, A. & Siess, W. Lysophosphatidic acid in atherosclerotic diseases. *Br. J. Pharmacol.* **167,** 465–82 (2012).
24. Lin, C. I., Chen, C.-N., Chen, J. H. & Lee, H. Lysophospholipids increase IL-8 and MCP-1 expressions in human umbilical cord vein endothelial cells through an IL-1-dependent mechanism. *J. Cell. Biochem.* **99,** 1216–1232 (2006).
25. Zhou, Z. *et al.* Lipoprotein-derived lysophosphatidic acid promotes atherosclerosis by releasing CXCL1 from the endothelium. *Cell Metab.* **13,** 592–600 (2011).
26. Lee, H. *et al.* Lysophospholipids increase ICAM-1 expression in HUVEC through a Gi- and NF-kappaB-dependent mechanism. *Am. J. Physiol. Cell Physiol.* **287,** C1657–66 (2004).
27. Zhou, Z. *et al.* Lipoprotein-derived lysophosphatidic acid promotes atherosclerosis by releasing CXCL1 from the endothelium. *Cell Metab.* **13,** 592–600 (2011).
28. Goetzl, E. J., Kong, Y. & Voice, J. K. Cutting edge: differential constitutive expression of functional receptors for lysophosphatidic acid by human blood lymphocytes. *J. Immunol.* **164,** 4996–4999 (2000).
29. Li, S., Xiong, C. & Zhang, J. ATX and LPA receptor 3 are coordinately up-regulated in lipopolysaccharide-stimulated THP-1 cells through PKR and SPK1-mediated pathways. *FEBS Lett.* **586,** 792–797 (2012).
30. Chou, C.-H. *et al.* Lysophosphatidic acid alters the expression profiles of angiogenic factors, cytokines, and chemokines in mouse liver sinusoidal endothelial cells. *PLoS One* **10,** e0122060 (2015).
31. Damirin, A. *et al.* Role of lipoprotein-associated lysophospholipids in migratory activity of coronary artery smooth muscle cells. *Am. J. Physiol. Heart Circ. Physiol.* **292,** H2513–22 (2007).
32. Chan, L. C. *et al.* LPA3 receptor mediates chemotaxis of immature murine dendritic cells to unsaturated lysophosphatidic acid (LPA). *J. Leukoc. Biol.* **82,** 1193–1200 (2007).
33. Tanikawa, T., Kurohane, K. & Imai, Y. Regulatory effect of lysophosphatidic acid on lymphocyte migration. *Biol. Pharm. Bull.* **33,** 204–208 (2010).
34. Brault, S. *et al.* Lysophosphatidic acid induces endothelial cell death by modulating the redox environment. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **292,** R1174–83 (2007).
35. Ohta, H. *et al.* Ki16425, a subtype-selective antagonist for EDG-family lysophosphatidic acid receptors. *Mol. Pharmacol.* **64,** 994–1005 (2003).
36. Serbina, N. V & Pamer, E. G. Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2. *Nat. Immunol.* **7,** 311–317 (2006).
37. Thornton, A. M. *et al.* Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. *J. Immunol.* **184,** 3433–3441 (2010).
38. Akimova, T., Beier, U. H., Wang, L., Levine, M. H. & Hancock, W. W. Helios expression is a marker of T cell activation and proliferation. *PLoS One* **6,** e24226 (2011).
39. Rizza, C. *et al.* Lysophosphatidic acid as a regulator of endothelial/leukocyte interaction. *Lab. Invest.* **79,** 1227–1235 (1999).
40. Getz, G. S. & Reardon, C. A. Do the Apoe-/- and Ldlr-/- Mice Yield the Same Insight on Atherogenesis? *Arteriosclerosis, thrombosis, and vascular biology* (2016). doi:10.1161/ATVBAHA.116.306874

41. Zhang, S. H., Reddick, R. L., Piedrahita, J. A. & Maeda, N. Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. *Science* **258,** 468–471 (1992).

42. Ishibashi, S., Goldstein, J. L., Brown, M. S., Herz, J. & Burns, D. K. Massive xanthomatosis and atherosclerosis in cholesterol-fed low density lipoprotein receptor-negative mice. *J. Clin. Invest.* **93,** 1885–1893 (1994).

43. Roselaar, S. E., Kakkanathu, P. X. & Daugherty, A. Lymphocyte populations in atherosclerotic lesions of apoE -/- and LDL receptor -/- mice. Decreasing density with disease progression. *Arterioscler. Thromb. Vasc. Biol.* **16,** 1013–1018 (1996).

44. Tani, M. *et al.* The influence of apoE-deficiency and LDL-receptor-deficiency on the HDL subpopulation profile in mice and in humans. *Atherosclerosis* **233,** 39–44 (2014).

45. Murphy, A. J. *et al.* ApoE regulates hematopoietic stem cell proliferation, monocytosis, and monocyte accumulation in atherosclerotic lesions in mice. *J. Clin. Invest.* **121,** 4138–4149 (2011).

46. Bi, X. *et al.* Myeloid cell-specific ATP-binding cassette transporter A1 deletion has minimal impact on atherogenesis in atherogenic diet-fed low-density lipoprotein receptor knockout mice. *Arterioscler. Thromb. Vasc. Biol.* **34,** 1888–1899 (2014).

47. Joyce, C. W. *et al.* The ATP binding cassette transporter A1 (ABCA1) modulates the development of aortic atherosclerosis in C57BL/6 and apoE-knockout mice. *Proc. Natl. Acad. Sci. U. S. A.* **99,** 407–412 (2002).

48. Hanna, R. N. *et al.* NR4A1 (Nur77) deletion polarizes macrophages toward an inflammatory phenotype and increases atherosclerosis. *Circ. Res.* **110,** 416–427 (2012).

49. Goetzl, E. J., Kong, Y. & Mei, B. Lysophosphatidic acid and sphingosine 1-phosphate protection of T cells from apoptosis in association with suppression of Bax. *J. Immunol.* **162,** 2049–2056 (1999).

50. Zheng, Y., Voice, J. K., Kong, Y. & Goetzl, E. J. Altered expression and functional profile of lysophosphatidic acid receptors in mitogen-activated human blood T lymphocytes. *FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol.* **14,** 2387–2389 (2000).

51. Chan, L. C. *et al.* LPA3 receptor mediates chemotaxis of immature murine dendritic cells to unsaturated lysophosphatidic acid (LPA). *J. Leukoc. Biol.* **82,** 1193–200 (2007).

52. Klingenberg, R. *et al.* Depletion of FOXP3+ regulatory T cells promotes hypercholesterolemia and atherosclerosis. *J. Clin. Invest.* **123,** 1323–1334 (2013).

53. Bagga, S. *et al.* Lysophosphatidic acid accelerates the development of human mast cells. *Blood* **104,** 4080–7 (2004).

54. Lundequist, A. & Boyce, J. A. LPA5 is abundantly expressed by human mast cells and important for lysophosphatidic acid induced MIP-1beta release. *PLoS One* **6,** e18192 (2011).

55. Rancoule, C. *et al.* Lysophosphatidic acid impairs glucose homeostasis and inhibits insulin secretion in high-fat diet obese mice. *Diabetologia* **56,** 1394–402 (2013).

56. Orosa, B. *et al.* Lysophosphatidic acid receptor inhibition as a new multipronged treatment for rheumatoid arthritis. *Ann. Rheum. Dis.* **73,** 298–305 (2014).

57. Tager, A. M. *et al.* The lysophosphatidic acid receptor LPA1 links pulmonary fibrosis to lung injury by mediating fibroblast recruitment and vascular leak. *Nat. Med.* **14,** 45–54 (2008).

58. Chomczynski, P. & Sacchi, N. The single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction: twenty-something years on. *Nat. Protoc.* **1,** 581–585 (2006).

Supplementary Information

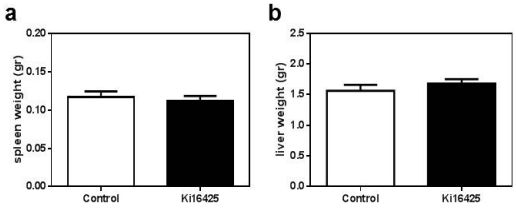


Figure 1: Treatment with Ki16425 does not affect the spleen or liver weight of mice. (a) The spleen weight between the control and Ki16425 treated group showed no significant difference (P=0.63) at the end of the study. **(b)** Similarly, the liver weight between the control and Ki16425 groups was not substantially different (P=0.38). All values (n=13/grp; spleen and n=6/grp; liver) are depicted as mean±SEM.

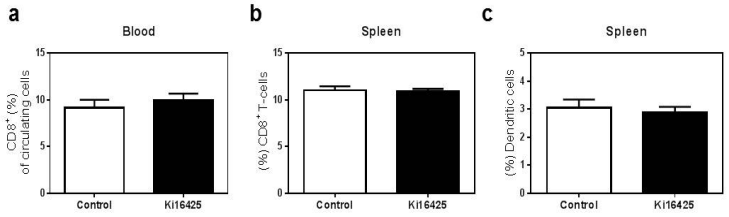


Figure 2: $LPA_{1/3}$ inhibition did not affect the amount of $CD8^+$ T lymphocytes or dendritic cells. The amount of $CD8^+$ T cells in the **(a)** circulation and **(b)** spleen was not affected by treatment with Ki16425 (P=0.50) and (P=0.78). **(c)** The overall percentage of dendritic cells present in the spleen showed no differences among the control and treated mice (P=0.60). All values (n=12/grp) are depicted as mean±SEM.

Gene	*Forward primer (5'-3')*	*Reverse primer (3'-5')*
CCL2	GCATCT GCCCTAAGGTCT TCA	TTCACTGTCACACTGGTCACTCCTA
CD68	TGCCTGACAAGGGACACTTCGGG	GCGGGTGATGCAGAAGGCGATG
CXCL1	TTGACCCTGAAGCTCCCTTG	AGGTGCCATCAGAGCAGTC
I-CAM 1	GTCCGCTTCCGCTACCATCA	GGTCCTTGCCTACTTGCTGCC
Rpl27	CGCCAAGCGATCCAAGATCAAGTCC	AGCTGGGTCCCTGAACACATCCTTG
β-actin	AACCGTGAAAAGATGACCCAGAT	CACAGCCTGGATGGCTACGTA

Supplementary Table 1: qPCR gene primer sequences. All gene expression analysis was performed using two housekeeping genes (β-actin and Rpl27). Abbreviations: Chemokine C-C motif ligand 2 (CCL2); cluster of differentiation 68 (CD68); chemokine C-X-C motif ligand 1 (CXCL1); Intercellular adhesion molecule-1 (ICAM-1); 60s ribosomal protein ligand 27 (Rpl27).

Antibody	Fluorochrome	Clone	Company
CD11b	eFluor 450	M1/70	Ebioscience
Ly6C	PercP Cy5.5	HK1.4	Ebioscience
Ly6G	PE	1A8	BD Biosciences
CCR2	APC	#475301	R&D Systems
CD4	PercP	Rm4-5	BD Biosciences
CD25	FITC	PC61.5	Ebioscience
NK1.1	FITC	PK136	Ebioscience
CD8a	PercP	53-6.7	BD Biosciences
MHC-II	eFluor 450	AF6-120.1	Ebioscience
CD11c	APC	N418	Ebioscience
FoxP3	*eFluor 450*	*FJK-16s*	Ebioscience
T-bet	*Alexa -Fluor 660*	*eBio4B10*	Ebioscience
Helios	*Alexa-Fluor 647*	*22F6*	Ebioscience

Supplementary Table 2: List of extracellular and *intracellular* antibodies used.

Chapter 4

Hypercholesterolemia induces a mast cell - CD4⁺ T cell interaction in atherosclerosis

Manuscript submitted for publication

[1]Eva Kritikou
[1]Thomas van der Heijden
[1]Maarten Swart
[1]Janine van Duijn
[1]Bram Slütter
[2]Anouk Wezel
[2]Harm J. Smeets
[3]Pasquale Maffia
[1]Johan Kuiper
[1]Ilze Bot

[1] Division of Biopharmaceutics, Leiden Academic Centre for Drug Research, Leiden University, Leiden, The Netherlands

[2]Department of Surgery. HMC Westeinde, The Hague, The Netherlands

[3]Centre for Immunobiology, University of Glasgow, United Kingdom

Abstract

Mast cells are potent innate immune cells that aggravate atherosclerosis through the release of pro-inflammatory mediators inside atherosclerotic plaques. Similarly, $CD4^+$ T cells are constituents of the adaptive immune response and accumulate within the plaques following lipid-specific activation by antigen presenting cells. Lately it is being proposed that these two cell types can interact, upon request, in a direct manner. However, no indication of such an interaction has been previously investigated in atherosclerosis. In our study, we aimed to examine whether mast cells can act as non-classical antigen presenting cells in atherosclerosis, thereby shaping the $CD4^+$ T cell response. We observed that mast cells can increase their MHC-II expression under hyperlipidemic conditions both *in vivo* and *in vitro*. Furthermore, we showed that mast cells can present antigens *in vivo via* their MHC-II molecules. High-fat diet serum enhanced the expression of the co-stimulatory molecule CD86 on the mast cell surface *in vitro,* while co-culture of OVA-peptide loaded mast cells shifted the OT-II $CD4^+$ T cell response towards a T_{H1} cell subset. In accordance, the aortic T_{H1} cell content of atherosclerotic mice that lack mast cells was found reduced as compared to their wild-type counterparts. Importantly, we here established that mast cells in advanced human atherosclerotic plaques express HLA-DR, indicating that they are capable of antigen presentation inside human atherosclerotic tissues. Therefore, we are the first to show that mast cells may function as non-classical antigen presenting cells and directly modulate adaptive immunity in atherosclerosis.

1. Introduction

Atherosclerosis is a chronic, lipid-mediated, autoimmune disease of the medium and large arteries, which involves both innate and adaptive immune reactions[1]. The communication between the innate and adaptive immune response is achieved through professional antigen presenting cells, such as dendritic cells, that can specifically activate T lymphocytes with antigens taken up from the atherosclerotic plaque[2]. It has previously been established that during the progression of atherosclerosis, antigens from the oxidized low-density lipoprotein (oxLDL) molecules can elicit immune responses by activating an array of T cell subtypes, like the T_{H1} and T_{H2} cell types[3,4]. This happens inside secondary lymphoid tissues, such as the spleen and the local draining lymph nodes and requires three key-signals that involve presentation of the antigenic fragments. Antigen presentation is triggered by major histocompatibility complex I or II (MHC-I/II) and followed by a strong co-stimulatory response, in the form of proteins like CD86, along with secreted cytokines priming the T cell activation process[5]. In response, activated T cells subsets translocate to the atherosclerotic plaque and influence the disease outcome locally[6]. The differential T cell subset effect is partially attributed to the different cytokine milieu secreted by the two cell types. Specifically, T_{H1} cells, which are the most abundant type inside progressing atherosclerotic plaques, secrete large amounts of interferon-γ (IFNγ) while T_{H2} cells are found at lower numbers and release mainly interleukin-4 (IL-4), interleukin-5 (IL-5) and interleukin-13 (IL-13)[7,8]. $CD4^+$ T_{H1} cells have been proven to be proatherogenic[9], while T_{H2} cells appear to counteract for the T_{H1}-mediated effects[10] and through secretion of IL-5 show an atheroprotective function. On the other hand, IL-4 cytokine is reported to have a pro-atherogenic function with lower plaques developing upon its absence[11] making thus the exact role of T_{H2} cells in atherosclerosis hard to define[12]. Therefore, the diversity, in terms of quality and quantity of the T cell subsets, can exert differential effects and inversely affect the progression of atherosclerosis. Still, although activated T cells have in principle a pre-established fate upon entering the vessel wall, local costimulation in the plaques[13,14] may alter their behaviour.

Mast cells are tissue resident innate immune cells that have long been established to contribute to atherosclerosis progression[15]. These cells accumulate in the atherosclerotic tissue upon plaque development and, according to their classical mode of action, release pro-inflammatory mediators such as proteases and cytokines[16,17]. Yet, increasing evidence suggests that mast cells can also adopt an inducible antigen presenting phenotype that may influence the immune response inside tissues[18]. While mast cells are not classical antigen presenting cells like dendritic cells, peritoneal mast cells have been found to upregulate MHC-II in the presence of cytokines IL-4 and IFNγ[19], or inside the lymph nodes of mice that have been injected with lipopolysaccharide (LPS)[20].

Importantly, mouse bone marrow derived mast cells have been reported to present peptides of the model-antigen ovalbumin (OVA) to $CD4^+$ T cells[21]. Likewise, human cultured mast cells can directly present antigens to $CD4^+$ T cells through the human MHC-II equivalent protein HLA-DR[22,23]. However, up to date it is not known whether mast cells can directly influence the adaptive immune response during atherosclerotic plaque progression. Therefore, we aimed to study the antigen presentation capacity of mast cells to $CD4^+$ T cells in atherosclerosis.

2. Materials & Methods

2.1 Animals

All animal work was performed in compliance with the guidelines directed by the European Union Directive 2010/63EU and the Dutch Government. The experimental work was approved by the Animal Ethics committee of Leiden University. The animals were originally obtained by the Jackson Laboratories and subsequently bred in the local animal facility, while provided with food and water *ad libitum*. At experimental endpoint, mice were subcutaneously anesthetized with an injection mix of ketamine (100mg/mL), sedazine (25mg/mL) and atropine (0.5mg/mL) and their vascular system was perfused with PBS upon heart puncture in the left ventricle.

2.2 WTD-timecourse experiment

In order to examine the effect of WTD on the antigen presentation capacity of mast cells we performed a timecourse experiment (**Supplemental Figure 1A**). Male $LDLr^{-/-}$ mice (n=10/grp) of an average age 8-10 weeks old were placed on a Western-type diet (WTD) (0.25% cholesterol, 15% cocoa butter; Special Diet Services, Essex, UK) for either 4 or 8 weeks, or kept on a chow diet until the experimental endpoint. Mouse peritoneal cells were collected by peritoneal lavage, using 10mL PBS, to study the mast cell peritoneal population according to their characteristic expression of markers FcεRIα and CD117. Notably, the peritoneal mast cell population was also examined for the expression of the myeloid cell marker CD11c, to ensure that the observed population was not $MHC\text{-}II^+$ dendritic cells or macrophage foam cells, as these cells have been previously reported to express inducible Fcε-receptors in atopic dermatitis, allergy and inside atherosclerotic plaques[24,25]. The para-aortic lymph nodes of these mice were isolated at the experimental endpoint and fixated in formalin for 24 hours. Subsequently, all lymph nodes were embedded in OCT medium (Sakura) and 6μm cryosections were collected. Mast cell detection was performed using a Naphthol AS-D chloro-acetate esterase kit (Sigma Aldrich). The T cell population in the para-aortic lymph nodes was detected through antibody staining against CD3 at a 1:150 concentration (clone SP7, Thermoscientific). Determination of all mast cell and $CD3^+$ T cell numbers was performed by blinded and independent manual quantification of 2 collected sections per mouse. Representative pictures were obtained using a Leica DM-RE microscope (Leica Systems, UK).

2.3 Eα-presentation experiment

In order to study the ability of mast cells to present antigens through their MHC-II molecule we made use of the Eα-peptide presentation system whereupon an Eα-GFP peptide, after internalization and degradation, can be presented by the MHC-II molecules on the surface of APCs and is detected by specifically designed antibodies against Eα-fragments[26] we performed an Eα-peptide presentation experiment (**Supplemental Figure 1B**). Male $LDLr^{-/-}$ mice (n=14/

grp, average age:13 weeks) were placed on a WTD for 4 weeks. 24 hours prior to the end of the experiment one group was injected intraperitoneally with Eα-GFP[27], (100μg/mouse) while an additional group was administered sterile PBS as a control. Peritoneal cells isolated from Eα-treated mice were further stimulated *ex vivo* with Eα-GFP (200μg/mL) for either 3 or 24 hours and compared with peritoneal cells isolated from the PBS injected mice. The expression levels of an Y-Ae-Streptavidin antibody against Eα were determined by flow cytometry in comparison to the control-PBS expression.

2.4 Bone marrow derived mast cell stimulation

To investigate the antigen presentation function of mast cells *in vitro*, bone marrow derived mast cells (BMMCs) were cultured from isolated bone marrow cells in RPMI 1640 medium containing 25mM HEPES (Lonza) supplemented with 10% fetal calf serum (FCS), (1x) minimal essential medium non-essential amino-acids (MEM NEAA; Gibco), 60μM β-mercaptoethanol (Sigma), 1% mix of penicillin/streptomycin (PAA), 1% sodium pyruvate (Sigma) and 2% L-glutamine (Lonza) for 4 weeks with cytokine IL-3 (5 ng/mL; Immunotools). Mast cell purity was assessed by the expression of FcεRIα and CD117 and routinely found to be above 98%. Mature mast cells kept in culture for weeks 4 - 6 were used for all experiments. MHC-II induced mast cells (iMCs) were obtained by a mix of cytokines IL-4 (20ng/mL) and IFNγ (50ng/mL). Subsequently, to study the effect of hyperlipidemic serum, iMCs were stimulated for 24h with 10% serum isolated from either chow fed or 4 weeks WTD fed $LDLr^{-/-}$ mice and all conditions were renewed for an additional period of 24h. iMCs were additionally stimulated with 40% peritoneal fluid which was obtained by peritoneal lavage with 10ml PBS and after centrifugation at 1.600 RPM for 5 minutes. After a period of 24h all conditions were renewed accordingly. Cytokines IL-12p70, IFN-γ and IL-4 secretion was measured by ELISA (BD Biosciences) according to the manufacturer's protocol.

2.5 Mast cell-OT-II CD4⁺ T cell co-culture

To study the direct presentation of antigens by mast cells to CD4⁺ T cells *in vitro* we made use of the OVA presentation system. In a co-culture experiment, iMCs were repeatedly treated with 10% chow/WTD serum, as described above, in the presence of an OVA-peptide (10μg/mL) that can directly bind the MHC-II surface molecules and is directly presented to the T cell receptor (TCR) without requiring internalization. CD4⁺ T cells were isolated from genetically modified OT-II mice[28] using a magnetic-bead isolation kit (Miltenyi) and activated with αCD3 (1μg/mL) and αCD28 (0.5μg/mL) for 24h. Subsequently, after washing off the stimulants, mast cells and T cells were placed together in a co-culture system. Cells were seeded in quintuplicate per condition at a 1:1 ratio for 72 hours. At the end of the experiment, all samples were prepared for flow cytometry. For the proliferation assay radioactive ^{3}H-Thymidine (0.5μCi/well; PerkinElmer) was added to the samples after 48h and the incorporation rate was measured 15h later using a liquid scintillation analyzer (Tri-Carb 2900R). The levels of cytokines IL-4 and IFNγ were determined by ELISA (BD Biosciences) according to the manufacturer's protocol.

2.6 Mast cell deficiency experiment

To investigate the effect of mast cells on the T cell population in atherosclerosis, male mast cell deficient $apoE^{-/-}/Kit^{W\text{-}sh/W\text{-}sh}$ mice as well as control $apoE^{-/-}$ mice (**Supplemental Figure 1C**) of (n=16/grp, average age 16 weeks), were fed a WTD for 6 weeks. The intraplaque cell content was obtained through enzymatic digestion of the aortic tissue for 30 minutes at 37°C, with a mix of collagenase I (450u/mL; Sigma) and XI (250U/mL Sigma) and DNase (120U/mL; Sigma), hyaluronidase (120 U/mL; Sigma) and samples were further processed using flow cytometry.

2.7 Human specimens

To explore the ability of human intraplaque mast cells to present antigens, 20 anonymous atherosclerotic plaques were collected post-operatively from carotid or femoral artery endarterectomy surgeries, performed between July 2016 and December 2016 at the Haaglanden Medical Center (HMC), Westeinde, The Hague, The Netherlands. The handling of all human samples was performed in accordance with the "Code for Proper Secondary Use of Human Tissue". All atherosclerotic samples were processed in single cell suspensions, as described previously[29]. In short, cell suspensions from human atherosclerotic plaques were obtained upon digestion with collagenase IV (Gibco) and DNase (Sigma) for 2 hours at 37°C prior to single-cell separation through a 70μm cell strainer. All human white blood cell populations were characterized by flow cytometry, based on the expression of the pan-leukocyte marker CD45. The mast cells were further defined by the FcεRIα and CD117 antibodies.

2.8 Flow cytometry

All cell populations are depicted as the percentage of viable cells, determined according to a fixable viability dye solution (eBioscience). Gating strategies and controls are shown in **Supplemental Figure 2**. Cells were stained with antibodies against extracellular proteins or fixated and permeabilized using a transcription factor kit (eBioscience) for intracellular stainings (**Table 1**). Flow cytometry measurements were performed on a FACS Canto II and data were analysed using FlowJo software.

Targeted Species	Antibody	Fluorochrome	Clone	Concentration	Company
Mouse/ Human	Fixable Viability Dye	eFluor 780	-	0.1 μg/sample	eBioscience
Mouse	FcεRIα	PercP Cy5.5	MAR-1	0.1 μg/sample	Biolegend
Mouse	CD117	APC/PE Cy7/ PE	2B8	0.1 μg/sample	eBioscience
Mouse	MHC-II	FITC/PE	M5/ 114.15.2	0.1 μg/sample	eBioscience
Mouse	CD11c	PE	N418	0.1 μg/sample	eBioscience
Mouse	CD86	PE/APC	GL1	0.1 μg/sample	eBioscience
Mouse	Ea52-68	Biotin	EBioY-Ae	0.5 μg/sample	eBioscience
Mouse	Streptavidin	APC	-	0.3 μg/sample	eBioscience
Mouse	Thy1.2	PE Cy7	53-2.1	0.1 μg/sample	eBioscience
Mouse	CD4	PercP	RM4-5	0.1 μg/sample	BD Biosciences
Mouse	CD8	eFluor 450	53-6.7	0.1 μg/sample	eBioscience
Mouse	*T-bet*	*Alexa-Fluor 660*	*eBio4B10*	*0.3 μg/sample*	eBioscience
Mouse	*Ki-67*	*FITC*	SolA15	*0.3 μg/sample*	eBioscience
Human	CD45	PE Cy7	2D1	0.3μg/sample	eBioscience
Human	FcεRIα	APC	AER-37	0.1μg/sample	eBioscience
Human	CD117	PercP Cy5.5	104D2	0.1μg/sample	BD Biosciences
Human	HLA-DR	PE	L243	0.3μg/sample	eBioscience
Human	CD63	PE	H5C6	0.1μg/sample	eBioscience

Table 1: List of extracellular and *intracellular* antibodies used in all flow cytometry experiments

2.9 Statistics

All data are presented as mean±SEM. Values within groups were tested for normal distribution and corrected with a Bonferroni post-test for multiple comparisons. In the event of one-variable analysis among two groups a 2-tailed Student's *t*-test was used, while among more than two groups a one-way ANOVA was performed. For the analysis of two variables between groups, a two-way ANOVA test was used. Non-Gaussian distributed data were analyzed with a Mann-Whitney *U* test. Pearson's correlation was used to estimate the association between two variables in human mast cells. The probability (alpha) value for all tests was set to 0.05, with differences lower than this considered significant ($P<0.05$).

3. Results

3.1 Western-type diet increases the MHC-II expression on peritoneal mast cells

To determine the effects of high lipid diet on the mast cells, we fed LDLr$^{-/-}$ mice a WTD for a period of 4 to 8 weeks and compared the peritoneal mast cell population with LDLr$^{-/-}$ mice fed a chow diet. Upon increasing periods of WTD, the relative amount of peritoneal mast cells increased significantly (**Figure 1A**, chow: 0.39±0.1% vs WTD 4wk: 1.97±0.4%, P=0.014; chow vs WTD 8wk: 2.02±0.3%, P=0.011). Likewise, the absolute number of peritoneal mast cells increased after 4 weeks of WTD (**Figure 1B**, P=0.060), however this number returned to chow-levels upon 8 weeks of WTD. Interestingly, we observed that during 4 weeks of WTD the peritoneal mast cell population showed an enhancement in expression of the antigen presenting protein MHC-II (**Figure 1C**, chow-MCs:30.38±1.7% vs WTD 4wk-MCs:52.48±5.9%, P=0.014). The absolute number of mast cells that express MHC-II upon 4 weeks of WTD showed a 4-fold increase as compared to the number of MHC-II^{+} MCs in chow diet (**Figure 1D**, chow: 3677±367 mast cells vs WTD 4wk: 21561±5071 mast cells, P=0.009). Also here, 8 weeks of WTD restored the amount of MHC-II^{+} expressing mast cells to chow-levels. Furthermore, the para-aortic lymph nodes of these mice showed a slight increase in the number of mast cells after 4 weeks of WTD, as compared to the chow-fed mice. The mast cells accumulated particularly in the subcapsular sinuses of the lymph nodes (**Supplemental Figures 3A**,**B**), an area where soluble antigens meet professional antigen presenting cells[30]. However, no difference was observed between the groups in the number of T cells in the area (**Supplemental Figure 3C**).

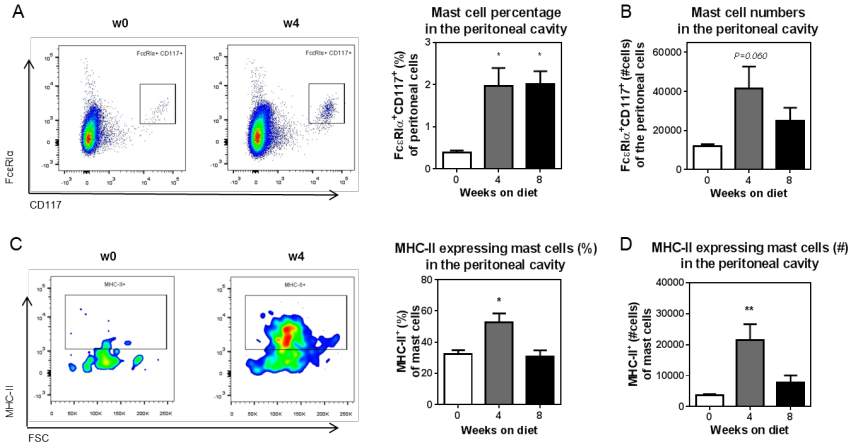


Figure 1: Peritoneal mast cells of LDLr$^{-/-}$ mice increase in amount and MHC-II expression after 4 weeks of WTD. Representative flow cytometry plots are shown, with the mast cell population defined by the expression of receptors FcεRIα and CD117. The mast cell percentage in the peritoneal cavity of LDLr$^{-/-}$ mice increased after 4 and 8 weeks of WTD (**A**). The mast cell numbers peaked after 4 weeks of WTD and were restored to normal levels after 8 weeks of WTD (**B**). Characteristic dot plots of the MHC-II expression on the mast cell population show the peritoneal mast cells that express protein MHC-II (**C**). The MHC-II-expressing mast cells increased significantly in percentage and numbers after 4 weeks of WTD, while returning to normal after 8 weeks (**D**). All values (n=5/gr) are depicted as mean±SEM; *$p<0.05$, **$p=0.01$

3.2 The MHC-II molecules on the peritoneal mast cells can present antigens *in vivo*

We next sought to examine whether the MHC-II molecules on the peritoneal mast cell surface are functional and capable of presenting antigens *in vivo* by using the Eα-peptide system[26]. We therefore injected the Eα-peptide or PBS-control in the peritoneum of WTD-fed LDLr$^{-/-}$ mice. After a period of 24 hours we observed that the MHC-II expression was enhanced in the Eα-injected mast cells as compared to control (**Figure 2A**, PBS:16.3±4.9% Eα:29.75±6.1% P=0.052). Importantly, Eα-peptide fragments were detected on the mast cell surface of the Eα-injected group (**Figure 2B**, PBS:2.73±0.3% vs Eα:7.07±1.5%, P=0.009). We further examined the capacity of peritoneal mast cells to present the Eα-peptide *ex vivo*. Upon 3h of peptide addition, Eα-GFP^{+} signal was detected in the peritoneal mast cells, indicating that the peptide is internalized but not yet processed by the lysosomal compartment (**Figure 2C**, PBS: 3.41±1.9% vs Eα(3h): 27.7±6.2%, P=0.008). No Eα-GFP^{+} cells were observed after 24 hours. However, at this timepoint Eα-peptide fragments were detected on the peritoneal mast cell surface, indicating that during this period the Eα-peptide was fully processed by the lysosomes and its fragments were presented on the mast cell surface through the MHC-II molecule (**Figure 2D**, PBS: 4.46±1.2% vs Eα(24h): 22.08±9.1%, P=0.062).

As expected, no fragment presentation by mast cells was detected in the first 3h of Eα-addition.

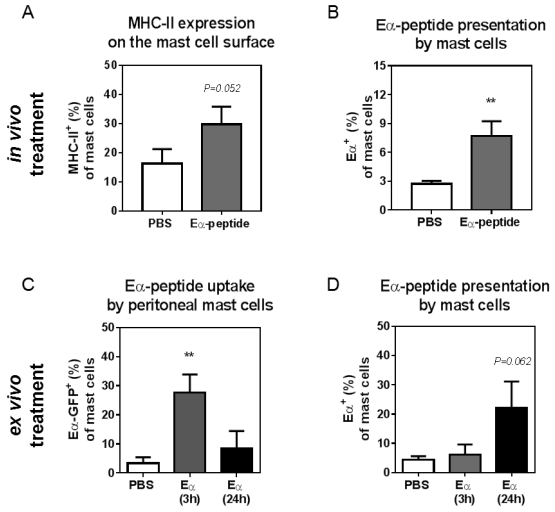


Figure 2: The MHC-II protein on the peritoneal mast cell surface is able to present antigenic fragments of the Eα-peptide, upon WTD. LDLr$^{-/-}$ mice, fed a WTD for 4 weeks, increase their MHC-II expression levels 24h after i/p injection of Eα-GFP, as compared to the PBS mice (n=10/grp) (**A**). The Eα-peptide fragments are detected *in vivo* on the surface of peritoneal mast cells, 24h after administration (n=11/grp) (**B**). Peritoneal cells isolated from the PBS and Eα-administered mice (n=6/grp) were further treated *ex vivo* with Eα-GFP peptide. Eα-GFP uptake by peritoneal mast cells was detected 3h after addition, while 24h later there was no detectable Eα-GFP signal (**C**). Eα-peptide fragments were observed 24h later, on the peritoneal mast cell surface (**D**). All values are depicted as mean±SEM (n=10/grp); ***p<0.01*.

3.3 WTD serum enhances the presentation capacity of bone marrow derived mast cells *in vitro*

Since WTD-induced hypercholesterolemia seemed to enhance the presentation machinery of mast cells, we aimed to further investigate the direct effect of hyperlipidemic serum on the non-classical MHC-II expression of mast cells. We therefore used bone-marrow derived mast cells (BMMCs) to examine the effects of atherogenic serum isolated from mice fed a WTD for 4 weeks or chow diet serum. It is worth mentioning here that, unlike peritoneal mast cells[31], bone marrow derived mast cells are not able to constitutively express MHC-II[32] unless induced with specific factors[19,20]. For that reason, in our BMMC experiments we induced MHC-II expression on the mast cells (iMCs) using IL-4 and IFNγ (**Supplemental Figure 4A**). This does not seem to be necessary *in vivo* because hyperlipidemic serum already contains these cytokines (**Supplemental Figure 4B**,**C**). We observed that upon repeated treatment of iMCs, for 48h with WTD (4wk) serum, mast cells increased their MHC-II expression levels by 2-fold as compared to chow serum (**Figure 3A**, chow-iMC: 3.12±0.3% vs WTD-iMC: 10.25±0.1%; P<0.0001). Furthermore, the costimulatory protein CD86, which is necessary for the strengthening of the antigen presentation process[33], was also found to increase in a WTD specific manner on the surface of the WTD-iMCs, as compared to chow-iMCs. (**Figure 3B**, chow-iMC: 1.73±0.1% vs WTD-iMC: 2.53±0.1%, P=0.0012). Finally, for antigen presentation to be efficient, cytokine signals are also needed. Therefore, we measured the levels of

cytokine IL-12, which is one of the most potent cytokines for T_{H1} subset skewing[34]. We did not observe any differences between the WTD and chow treated iMCs (**Figure 3C**), however it was certain that mast cells, in the presence of serum are able to provide the third signal needed for $CD4^+$ T_{H1} cell skewing. Interestingly, when iMCs were treated with fluid collected from the peritoneal cavity of $LDL^{-/-}$ mice fed either chow or WTD (4 weeks) they showed a marked increase in their MHC-II expression (**Supplemental Figure 4D**). We therefore measured the IL-4 and IFNγ levels in the peritoneal cell fluid and observed that both cytokines are present (**Supplemental Figure 4E**,**F**), indicating that peritoneal mast cells seem to have the necessary signals needed for MHC-II induction *in vivo*.

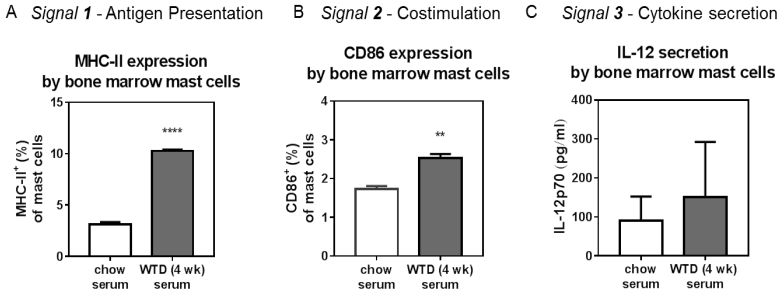


Figure 3: Inducible bone marrow derived mast cells repeatedly treated with WTD serum possess all three signals required for antigen presentation. The expression of MHC-II on the surface of bone marrow mast cells increased upon repeated (48h) *in vitro* treatment with 10% serum isolated from $LDLr^{-/-}$ mice that were fed a WTD for 4 weeks, as compared to chow serum (**A**). Co-stimulatory molecule CD86 was enhanced on the surface of mast cells after repeated treatment with 10% WTD (4wk) serum from $LDLr^{-/-}$ in comparison to chow-serum (**B**). Bone marrow mast cells were capable of secreting cytokine IL-12 after repeated serum treatment, with no significant difference detected between chow or WTD samples (**C**). All values are shown as mean±SEM; *$p<0.05$, **$p<0.01$.

3.4 OVA-peptide specific $CD4^+$ T cell proliferation is enhanced upon co-culture with WTD-treated mast cells

Thereafter, we aimed to unravel the direct effect of mast cell mediated antigen presentation to $CD4^+$ T cells. We isolated $CD4^+$ T cells from genetically modified OT-II mice, that specifically recognize ovalbumin antigens[28], and after 24h of pre-activation with αCD3/CD28 we placed them in a coculture with iMCs loaded with an OVA-peptide (OVAp) in the presence of either chow or WTD diet. OVAp-loaded iMCs showed a significant increase on their MHC-II expression in the presence of $CD4^+$ T cells, regardless of the type of serum (**Figure 4A**, $P<0.001$). Furthermore, the OVAp-iMC population pretreated with WTD-serum showed an increase in their CD86 expression when co-cultured with $CD4^+$ T cells (**Figure 4B**). $CD4^+$ T cells increased their proliferation rate in an OVAp specific manner with significant enhancement in the proliferation rate when co-cultured with WTD-pretreated iMCs, as detected by

the incorporation rate of ^{3}H-Thymidine (**Figure 4C**, chow-iMCs:9033±2558 dpm vs WTD-iMCs: 25331±2889 dpm, P<0.0001). The WTD effect of OVAp-carrying iMCs on the proliferation rate of CD4⁺ T cells was further confirmed by flow cytometry after detection of the intracellular proliferation marker Ki-67 on the CD4⁺ T cell population (**Figure 4D**, chow-iMCs:17.76±0.09% vs WTD-iMCs:25.56±1.7%, P=0.004). Finally, we measured the secretion of IFNγ and IL-4 in the co-culture since, aside from inducing MHC-II on the mast cells, they are the most important cytokine "fingerprints" of the CD4⁺ T_{H1} or T_{H2} subsets respectively. The secretion of IFNγ was slightly increased in the WTD-iMC conditions upon the presence of CD4⁺ T cells; however, no difference was observed between the OVAp-induced IFNγ secretion by CD4⁺ T cells (**Figure 4E**). In contrast, IL-4 secretion was significantly increased in the chow iMC conditions, effect which was not observed in the WTD iMC-CD4⁺ samples, resulting thus in a significant difference in the IL-4 levels between chow and WTD conditions (**Figure 4F**, chow-iMC:14.16±2.3 pg/ml vs WTD-iMC:5.85±1.9 pg/ml, P=0.002) suggesting that mast cell induced antigen presentation in hyperlipidemic conditions favors the secretion of IFNγ over IL-4 and therefore T_{H1} cell skewing over T_{H2}.

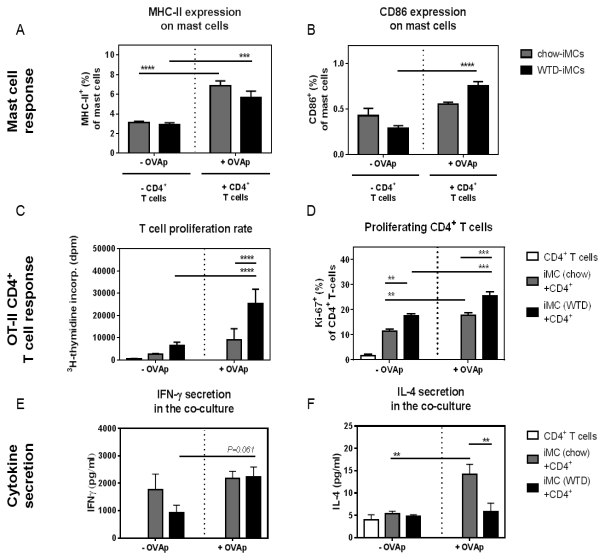


Figure 4: Bone marrow iMCs, repeatedly treated with WTD-serum and loaded with an OVA-peptide, enhance the proliferation rate of pre-activated OT-II CD4⁺ T cells. The expression of MHC-II on the mast cell surface was increased in OVAp-loaded mast cells at the presence of CD4⁺ T cells (**A**). The percentage of costimulatory molecule CD86 was found increased only WTD-treated mast cells that were loaded with OVAp and in the presence of CD4⁺ T cells (**B**). The proliferation rate of pre-activated OT-II CD4⁺ T cells increases upon presentation of OVAp by mast cells treated with WTD as compared to chow-treated MCs, as measured by both radioactive thymidine incorporation and expression of proliferation marker ki-67⁺ with flow cytometry (**C** and **D**). The IFNγ secretion by CD4⁺ T cells was increased upon presentation of the OVAp by WTD-treated mast cells as compared (**E**). The secretion of IL-4 by CD4⁺ T cells was increased upon presentation of OVAp by the chow-treated mast cells (**F**). All values (n=5/grp) are shown as mean±SEM; *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

3.5 Aortic CD4$^+$ T cells show reduced proliferation in mast cell deficient mice

To verify whether mast cells affect the CD4$^+$ T cell population in the atherosclerotic plaque we placed mast cell deficient apoE$^{-/-}$/Kit$^{W\text{-}sh/W\text{-}sh}$ mice on a WTD and compared the intraplaque CD4$^+$ T cell content in their aortas with that from WTD-fed control apoE$^{-/-}$ mice. Using flow cytometry, we observed a reduction in the percentage of Ki-67$^+$ CD4$^+$ T cells in the aortas of atherosclerotic mast cell deficient mice as compared to controls (**Figure 5A**, apoE$^{-/-}$: 30.91±5.2 % vs apoE$^{-/-}$/Kit$^{W\text{-}sh/W\text{-}sh}$:13.7±1.2, P=0.013). This reduction was also apparent when comparing the absolute number or proliferating CD4$^+$ T cells (**Figure 5B**, apoE$^{-/-}$: 1725±567 #cells vs apoE$^{-/-}$/Kit$^{W\text{-}sh/W\text{-}sh}$: 389±59 #cells, P=0.037). Interestingly, a decline in the number of T_{H1} cells was also detected in the aortas of mast cell deficient apoE$^{-/-}$ mice as compared to the control apoE$^{-/-}$ mice (**Figure 5C**, apoE$^{-/-}$: 148±25 #cells vs apoE$^{-/-}$/Kit$^{W\text{-}sh/W\text{-}sh}$: 45±16 #cells, P=0.056). Of note, the total T cell percentage is found slightly reduced in the aortas of the mast cell deficient apoE$^{-/-}$ (**Figure 5D**). This reduction is not due to the CD8$^+$ T cell population which remains unchanged (**Figure 5E**) but rather due to the reduction of the CD4$^+$ T cells (**Figure 5F**, apoE$^{-/-}$: 4.72±0.8 % vs apoE$^{-/-}$/Kit$^{W\text{-}sh/W\text{-}sh}$: 2.37±0.7 #cells, P=0.028).

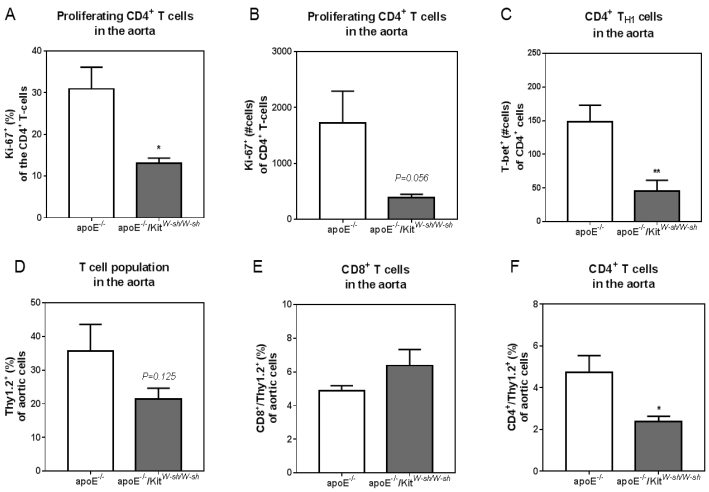


Figure 5: Mast cell deficient apoE$^{-/-}$/Kit$^{W\text{-}sh/W\text{-}sh}$ mice show a reduction in the proliferation of CD4$^+$ T_{H1} cells in the aortic arch. The percentage and absolute numbers of proliferating ki-67$^+$/CD4$^+$ T cells were reduced in the aortic arch of apoE$^{-/-}$/Kit$^{W\text{-}sh/W\text{-}sh}$ mice, fed a WTD for 6 weeks, as compared to apoE$^{-/-}$ mice (**A** and **B**). The numbers of T_{H1} cells in the aorta of mast cell deficient apoE$^{-/-}$ mice were decreased, as measured by the expression of transcription factor T-bet on the aortic CD4$^+$ T cell population (**C**). Mast cell deficient apoE$^{-/-}$ mice fed a WTD show a slight reduction in their aortic T cell percentage (**D**). The aortic CD8$^+$ T cell content of apoE$^{-/-}$ mice was not affected by the absence of mast cells (**E**). The aortic CD4$^+$ T cell content was significantly decreased in of apoE$^{-/-}$ mice fed a WTD upon the absence of mast cells (**F**). All values (n=8/group) are depicted as mean±SEM; *$p<0.05$, **$p<0.01$.

3.6 Human intraplaque mast cells express HLA-DR

Finally, to establish whether our data can be translated to the human disease setting, we analyzed the mast cell content of 20 endarterectomy plaques collected from carotid or femoral arteries. We are the first to use flow cytometry for the detection of human intraplaque mast cells, in femoral as well as carotid arteries as measured by the expression of receptors FcεRIα and CD117(**Figure 6A**, carotid hMCs: 0.81±0.1%, femoral hMCs: 1.26±0.2%). Focusing on the antigen presentation capacity of mast cells inside the atherosclerotic plaques, we further stained them for the expression of protein HLA-DR. We observed that mast cells inside human atherosclerotic plaques can express HLA-DR in both the femoral and the carotid arteries (**Figure 6B**, carotid hMCs: 9.30±4.3% vs femoral hMCs: 23.8±6.9%). Interestingly, the HLA-DR expressing cells showed a negative association with the activated mast cell population, as detected by marker CD63[35], which comprises the majority of mast cells within the atherosclerotic plaque (**Figure 6C**, P=0.024).

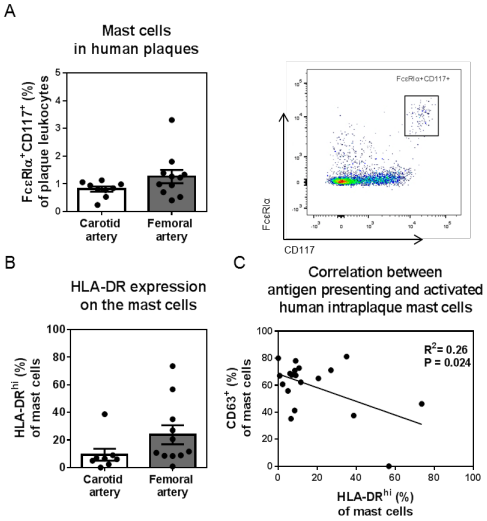


Figure 6: Human mast cells from carotid and femoral atherosclerotic plaques express HLA-DR. The mast cell population in the plaques of human subjects (n=20), obtained from endarterectomy surgery, are detected based on the high expression of FcεRIα and CD117. Representative flow cytometry plots of human mast cells, gated according to the expression of receptors FcεRIα and CD117 (**A**). Human intraplaque mast cells express HLA-DR, in the femoral artery and carotid artery samples (**B**). The HLA-DR^{hi} mast cells show a negative correlation with the activated CD63^{+} mast cell population in the atherosclerotic arteries (**C**).

4. Discussion

In this project, we sought to examine whether mast cells can act as non-professional antigen presenting cells in atherosclerosis. We observed that hyperlipidemic conditions induce the expression of MHC-II on the peritoneal mast cell surface and furthermore, that mast cells are able to process and present antigens *in vivo*. Within the atherosclerotic tissue, mast cells appear to augment the T_{H1} response in a manner that seems to partially involve a direct interaction between these two cell types. Importantly, we show

that antigen presentation by mast cells may even take place within the atherosclerotic plaques of human subjects, a process that appears to associate in a negative manner with the classical mast cell activation taking place within the plaque.

The capacity of mast cells to directly shape T cell responses, upon request by certain signals in their microenvironment, is a process that has recently gained attention in the field[36]. However, the question was posed long ago in a study reporting that rat mast cells incubated with IFNγ showed increased MHC-II expression; thus arguing that mast cells, aside from their classical degranulating nature may also be capable of presenting antigens[37]. The regulation of the MHC-II molecule expression by IFNγ has previously been established in professional antigen presenting cells[38], but also in human mast cells[39]. Additionally, peritoneal mast cells, in the presence of IFNγ/IL-4[19], have been shown to increase their MHC-II expression levels. This effect may explain why mast cells increase their MHC-II levels in an atherosclerotic environment, since it is known that patients with coronary heart disease show increased levels of IFNγ in their serum[40]. Moreover, human plaque tissue contains high amounts of IFNγ[41]. Here we observed that mast cells increase their MHC-II levels both *in vivo* and *in vitro* in a WTD-specific manner in accordance to detecting high levels of IFNγ, in the serum and peritoneal fluid of hyperlipidemic mice. We also saw that the absence of mast cells significantly reduced the number of T_{H1} cells, the main producers of IFNγ, inside the atherosclerotic plaques. Furthermore, antigen presentation through WTD-treated mast cells increased the IFNγ secretion by T cells, which, when combined with the reduced IL-4 secretion, supports the generation of T_{H1} cells. Mast cells have also been found to enhance plaque progression in an IFNγ dependent manner[16], with mast cell deficient mice developing smaller atherosclerotic plaques[42] which suggests that IFNγ may also function as a regulator of the direct interaction between mast cells and T_{H1} cells within the atherosclerotic plaque. It is worth noting that while WTD-treated mast cells seem to support a T_{H1} response, chow-treated mast cells tend to favor the T_{H2} phenotype. Interestingly, allergic asthma is a classic T_{H2} mediated condition where mast cells are famously known for their adverse effects[43]. In contrast, atherosclerosis is shaped by the action of T_{H1} cells, as mentioned before. Therefore, it seems that mast cells can act on the adaptive immune system *in situ* and according to the local inflammatory status.

Our study also shows that mast cells can express, upon request, all three signals required for antigen presentation. The presence of multiple costimulatory molecules on the mast cell surface, such as OX40L, CD80 and CD86, has been previously stated to be a main assistant in their direct communication with $CD4^+$ T cells[20,44]. Here we observed that WTD conditions increase CD86, however we cannot exclude that other costimulatory molecules may participate in the described interaction with $CD4^+$ T cells. Furthermore, mast cells are known for their potent cytokine secretion, and TNFα

release has also been reported to enhance local antigen presentation[32]. In the case of atherosclerosis, signal 3 could be provided by the mast cells, for instance in the form of IL-12 which skews T cells towards a T_{H1} subset, but also by the inflammatory cytokine content of the hypercholesterolemic serum.

In addition, we here establish that the MHC-II protein on the surface of mast cells is a fully functional molecule that can bind antigens processed by the lysosomal machinery of the cell and successfully transport them to the cell surface where they can be introduced to CD4⁺ T cells. However, while the Eα and OVA peptides are model antigens that serve in exploring the presentation capacity of a cell, the WTD-specific induction of MHC-II implies that lipid specific antigens may be presented by mast cells. Although it is still unclear which peptide fragments elicit an adaptive immune response in atherosclerosis it is worth mentioning that oxLDL has been previously reported to regulate the expression of MHC-II on macrophages in an autophagy mediated pathway[45]. It would therefore be intriguing to see if such a pathway is shaping also the lipid-specific mast cell presentation capacity that we observe here. Overall, it is interesting to investigate the means through which mast cells take up antigens from their surrounding atherosclerotic environment since, to our knowledge, mast cells are not capable of ingesting lipid proteins through the classic scavenger receptors such as those that mediate macrophage foam cell formation.

The way by which mast cells are activated within the atherosclerotic tissue has not yet been fully deciphered. While the classical FcεR-pathway is the most plausible manner, it is known that oxLDL can activate human mast cells in a Toll-like receptor (TLR)4-dependent mode[46]. This is of importance considering that TLR4 signaling on APCs enhances antigen presentation[47]. In line with the above, CD63 is an activation marker linked to the FcεR-mediated degranulation[35]. Notably, this study is the first to use flow cytometry as a method to detect human mast cells within atherosclerotic plaques. While we saw that intraplaque mast cells express high levels of CD63, their expression was negatively correlated to the HLA-DR expressing population. Even though this does not indicate a causal relationship, it seems that mast cells that are engaged with the presentation of antigens are not at that current moment activated. Furthermore, HLA-DR expression by human mast cells has also been previously reported in human tonsils[22], a common centre of inflammation in the body. Importantly, a very recent study investigating the presentation capacity of human skin mast cells provides additional evidence that these cells express HLA-DM in an IFNγ-specific manner and directly present antigens to CD4⁺ T cells further shaping thus not only the T_{H1} response[48], but also prompting T cells to act back on the mast cells. Likewise, we saw that CD4⁺ T cells act on the mast cell MHC-II expression in a positive way. It therefore seems that T cells can in turn affect the mast cells in a manner that favors the induced antigen presentation

ability of the latter.

Lastly, it is worth to mention that *in vitro* examination of the mast cell antigen presentation capacity in different studies shows slight discrepancies. This diversity probably lies on the *in vitro* generation of mast cells from bone marrow progenitors and the stimuli used. It is however important to remember that mast cells are found only within tissues, whereupon they undergo the last step of differentiation according to local stimuli.

In conclusion, this study showed that mast cells are capable of presenting antigens in a hypercholesterolemic manner and this effect is potently intensifying the proatherogenic T_{H1} cell type within atherosclerotic plaques. Importantly, mast cells seem capable of antigen presentation within human plaques, indicating that these cells can also shape the adaptive immune response apart from exacerbating the innate inflammatory pathways. Further research on how to modulate this interaction may induce novel ways to limit atherosclerosis development.

Reference list:

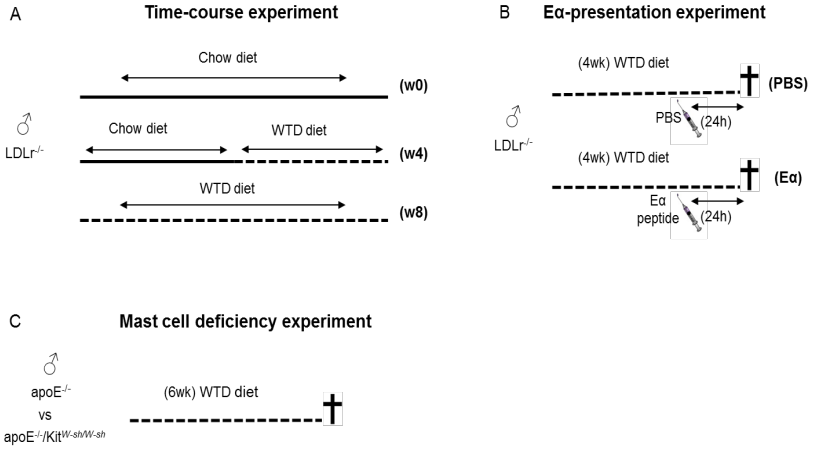
1. Gistera, A. & Hansson, G. K. The immunology of atherosclerosis. *Nat. Rev. Nephrol.* **13,** 368–380 (2017).
2. Zernecke, A. Dendritic cells in atherosclerosis: evidence in mice and humans. *Arterioscler. Thromb. Vasc. Biol.* **35,** 763–770 (2015).
3. Stemme, S. *et al.* T lymphocytes from human atherosclerotic plaques recognize oxidized low density lipoprotein. *Proc. Natl. Acad. Sci. U. S. A.* **92,** 3893–3897 (1995).
4. Libby, P., Lichtman, A. & Hansson, G. Immune effector mechanisms implicated in atherosclerosis: from mice to humans. *Immunity* **38,** 1092–1104 (2013).
5. Vega-Ramos, J., Roquilly, A., Asehnoune, K. & Villadangos, J. A. Modulation of dendritic cell antigen presentation by pathogens, tissue damage and secondary inflammatory signals. *Curr. Opin. Pharmacol.* **17,** 64–70 (2014).
6. Tse, K., Tse, H., Sidney, J., Sette, A. & Ley, K. T cells in atherosclerosis. *Int. Immunol.* **25,** 615–622 (2013).
7. Jonasson, L., Holm, J., Skalli, O., Bondjers, G. & Hansson, G. K. Regional accumulations of T cells, macrophages, and smooth muscle cells in the human atherosclerotic plaque. *Arteriosclerosis* **6,** 131–138 (1986).
8. Ketelhuth, D. F. J. & Hansson, G. K. Cellular immunity, low-density lipoprotein and atherosclerosis: break of tolerance in the artery wall. *Thromb. Haemost.* **106,** 779–786 (2011).
9. Laurat, E. *et al.* In vivo downregulation of T helper cell 1 immune responses reduces atherogenesis in apolipoprotein E-knockout mice. *Circulation* **104,** 197–202 (2001).
10. Buono, C. *et al.* T-bet deficiency reduces atherosclerosis and alters plaque antigen-specific immune responses. *Proc. Natl. Acad. Sci. U. S. A.* **102,** 1596–1601 (2005).
11. King, V. L., Szilvassy, S. J. & Daugherty, A. Interleukin-4 deficiency decreases atherosclerotic lesion formation in a site-specific manner in female LDL receptor-/- mice. *Arterioscler. Thromb. Vasc. Biol.* **22,** 456–461 (2002).
12. Aukrust, P. *et al.* The complex role of T-cell-based immunity in atherosclerosis. *Curr. Atheroscler. Rep.* **10,** 236–243 (2008).
13. Grivel, J.-C. *et al.* Activation of T lymphocytes in atherosclerotic plaques. *Arterioscler. Thromb. Vasc. Biol.* **31,** 2929–2937 (2011).
14. Koltsova, E. K. *et al.* Dynamic T cell-APC interactions sustain chronic inflammation in atherosclerosis. *J. Clin. Invest.* **122,** 3114–3126 (2012).
15. Bot, I., Shi, G.-P. & Kovanen, P. T. Mast cells as effectors in atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* **35,** 265–271 (2015).
16. Sun, J. *et al.* Mast cells promote atherosclerosis by releasing proinflammatory cytokines. *Nat. Med.* **13,** 719–724 (2007).
17. Ramalho, L. S. *et al.* Role of mast cell chymase and tryptase in the progression of atherosclerosis: study in 44 autopsied cases. *Ann. Diagn. Pathol.* **17,** 28–31 (2013).
18. Kambayashi, T. & Laufer, T. M. Atypical MHC class II-expressing antigen-presenting cells: can anything replace a dendritic cell? *Nat. Rev. Immunol.* **14,** 719–730 (2014).
19. Gaudenzio, N. *et al.* Cell-cell cooperation at the T helper cell/mast cell immunological synapse. *Blood* **114,** 4979–4988 (2009).
20. Kambayashi, T. *et al.* Inducible MHC class II expression by mast cells supports effector and regulatory T cell activation. *J. Immunol.* **182,** 4686–4695 (2009).
21. Gong, J. *et al.* The antigen presentation function of bone marrow-derived mast cells is spatiotemporally restricted to a subset expressing high levels of cell surface FcepsilonRI and MHC II. *BMC Immunol.* **11,** 34 (2010).

22. Suurmond, J. *et al.* Communication between human mast cells and CD4(+) T cells through antigen-dependent interactions. *Eur. J. Immunol.* **43,** 1758–1768 (2013).
23. Stern, L. J. & Calvo-Calle, J. M. HLA-DR: molecular insights and vaccine design. *Curr. Pharm. Des.* **15,** 3249–3261 (2009).
24. Tunon-De-Lara, J. M. *et al.* Dendritic cells in normal and asthmatic airways: expression of the alpha subunit of the high affinity immunoglobulin E receptor (Fc epsilon RI -alpha). *Clin. Exp. Allergy* **26,** 648–655 (1996).
25. Wang, J. *et al.* IgE stimulates human and mouse arterial cell apoptosis and cytokine expression and promotes atherogenesis in Apoe-/- mice. *J. Clin. Invest.* **121,** 3564–3577 (2011).
26. AYu, R., Rath, S., Preston-Hurlburt, P., Murphy, D. B. & Janeway, C. A. J. On the complexity of self. *Nature* **353,** 660–662 (1991).
27. Maddaluno, M. *et al.* Murine aortic smooth muscle cells acquire, though fail to present exogenous protein antigens on major histocompatibility complex class II molecules. *Biomed Res. Int.* **2014,** 949845 (2014).
28. Robertson, J. M., Jensen, P. E. & Evavold, B. D. DO11.10 and OT-II T cells recognize a C-terminal ovalbumin 323-339 epitope. *J. Immunol.* **164,** 4706–4712 (2000).
29. Van Brussel, I. *et al.* Fluorescent activated cell sorting: an effective approach to study dendritic cell subsets in human atherosclerotic plaques. *J. Immunol. Methods* **417,** 76–85 (2015).
30. Sainte-Marie, G. The lymph node revisited: development, morphology, functioning, and role in triggering primary immune responses. *Anat. Rec. (Hoboken).* **293,** 320–337 (2010).
31. Fox, C. C., Jewell, S. D. & Whitacre, C. C. Rat peritoneal mast cells present antigen to a PPD-specific T cell line. *Cell. Immunol.* **158,** 253–264 (1994).
32. Nakae, S. *et al.* Mast cells enhance T cell activation: importance of mast cell costimulatory molecules and secreted TNF. *J. Immunol.* **176,** 2238–2248 (2006).
33. Bour-Jordan, H. & Blueston, J. A. CD28 function: a balance of costimulatory and regulatory signals. *J. Clin. Immunol.* **22,** 1–7 (2002).
34. Teng, M. W. L. *et al.* IL-12 and IL-23 cytokines: from discovery to targeted therapies for immune-mediated inflammatory diseases. *Nat. Med.* **21,** 719–729 (2015).
35. Kraft, S. *et al.* The tetraspanin CD63 is required for efficient IgE-mediated mast cell degranulation and anaphylaxis. *J. Immunol.* **191,** 2871–2878 (2013).
36. Bulfone-Paus, S. & Bahri, R. Mast Cells as Regulators of T Cell Responses. *Front. Immunol.* **6,** 394 (2015).
37. Banovac, K., Neylan, D., Leone, J., Ghandur-Mnaymneh, L. & Rabinovitch, A. Are the mast cells antigen presenting cells? *Immunol. Invest.* **18,** 901–906 (1989).
38. Giroux, M., Schmidt, M. & Descoteaux, A. IFN-gamma-induced MHC class II expression: transactivation of class II transactivator promoter IV by IFN regulatory factor-1 is regulated by protein kinase C-alpha. *J. Immunol.* **171,** 4187–4194 (2003).
39. Love, K. S., Lakshmanan, R. R., Butterfield, J. H. & Fox, C. C. IFN-gamma-stimulated enhancement of MHC class II antigen expression by the human mast cell line HMC-1. *Cell. Immunol.* **170,** 85–90 (1996).
40. Liang, K., Dong, S.-R. & Peng, H. Serum levels and clinical significance of IFN-gamma and IL-10 in patients with coronary heart disease. *Eur. Rev. Med. Pharmacol. Sci.* **20,** 1339–1343 (2016).
41. Edsfeldt, A. *et al.* Circulating cytokines reflect the expression of pro-inflammatory cytokines in atherosclerotic plaques. *Atherosclerosis* **241,** 443–449 (2015).
42. Smith, D. D. *et al.* Mast cell deficiency attenuates progression of atherosclerosis and

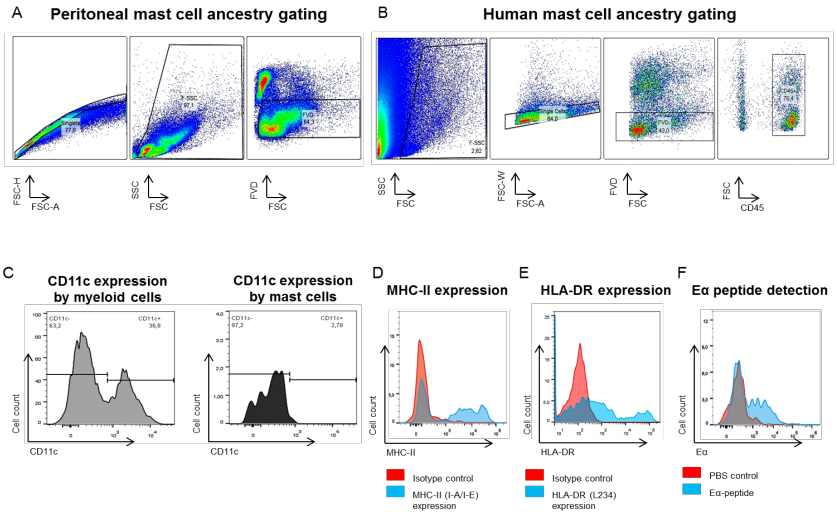
hepatic steatosis in apolipoprotein E-null mice. *Am. J. Physiol. Heart Circ. Physiol.* **302,** H2612-21 (2012).

43. Georas, S. N., Guo, J., De Fanis, U. & Casolaro, V. T-helper cell type-2 regulation in allergic disease. *Eur. Respir. J.* **26,** 1119–1137 (2005).
44. Nakano, N. *et al.* Notch signaling confers antigen-presenting cell functions on mast cells. *J. Allergy Clin. Immunol.* **123,** 74–81.e1 (2009).
45. Choi, S.-H. *et al.* SYK regulates macrophage MHC-II expression via activation of autophagy in response to oxidized LDL. *Autophagy* **11,** 785–795 (2015).
46. Meng, Z. *et al.* Oxidized low-density lipoprotein induces inflammatory responses in cultured human mast cells via Toll-like receptor 4. *Cell. Physiol. Biochem.* **31,** 842–53 (2013).
47. Mantegazza, A. R. *et al.* TLR-dependent phagosome tubulation in dendritic cells promotes phagosome cross-talk to optimize MHC-II antigen presentation. *Proc. Natl. Acad. Sci. U. S. A.* **111,** 15508–15513 (2014).
48. Lotfi-Emran, S. *et al.* Human mast cells present antigen to autologous CD4+ T Cells. *J. Allergy Clin. Immunol.* (2017). doi:10.1016/j.jaci.2017.02.048

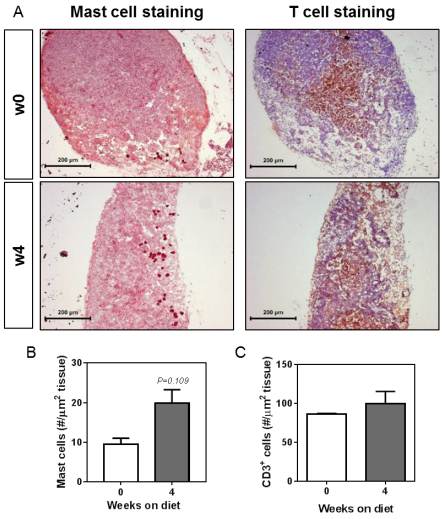
Supplementary Information



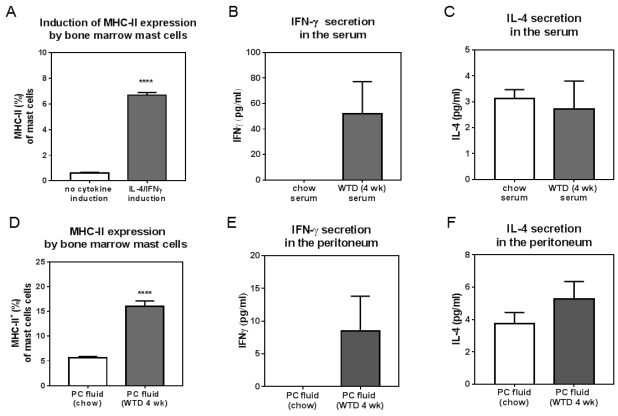
Supplemental Figure 1: **Experimental set-up for the *in vivo* experiments.** Time-course experiment using LDLr$^{-/-}$ mice fed 4 to 8 weeks Western-type diet or chow diet fed mice to compare their peritoneal mast cell populations (**A**). Eα-peptide presentation experiment performed on LDLr$^{-/-}$ mice fed a WTD for 4 weeks. Eα-peptide or sterile PBS was injected 24 hours prior sacrifice, in order to analyze the presentation capacity of peritoneal mast cells (**B**). Analysis of the aortic CD4^{+} T cell content in mast cell deficient apoE$^{-/-}$ mice, as compared to their wild-type counterparts, upon WTD feeding (**C**).



Supplemental Figure 2: **Gating strategies & controls of flow cytometry experiments.** Representative ancestry gating of mouse peritoneal mast cells and human intraplaque mast cells discriminated for their singularity based on cell height (FSC-H) or width (FSC-W) and area (FSC-A) as well as size (FSC) and granularity (SSC). The cells were further separated from the dead population which stained positive for a viability marker (FVD) (**A**) and human cells were further stained for the pan-leukocyte marker (CD45) (**B**). Peritoneal mast cell population did not show any CD11c expression in comparison to peritoneal myeloid cells (**C**). Isotype control for MHC-II antibody clone (I-A/I-E) and HLA-DR antibody clone used in flow cytometry experiments (**D** and **E**). Eα-peptide expression on Eα-injected mice as compared to their PBS counterparts (**F**).



Supplemental Figure 3: **Mast cells increase in numbers inside the para-aortic draining lymph nodes of LDLr$^{-/-}$ upon 4 weeks of Western-type diet.** Representative pictures of para-aortic lymph nodes of LDLr$^{-/-}$ mice stained with Naphthol-chloroacetate esterase for mast cells (red) and with αCD3^{+} for T cells (brown) (**A**). The mast cell numbers were found increased in the para-aortic lymph nodes of LDLr$^{-/-}$ mice upon 4 weeks of WTD (**B**). The T cell numbers within the para-aortic lymph nodes of LDLr$^{-/-}$ were not altered upon 4 weeks of WTD as compared to control chow diet (**C**). All values (n=3/grp) are depicted as mean±SEM.



Supplemental Figure 4: Cytokine mediated induction of MHC-II on the mast cell surface: Bone marrow mast cells express MHC-II only upon induction by cytokines IL-4 (10ng/ml) and IFNγ (20ng/ml) (**A**). WTD serum isolated by LDLr$^{-/-}$ mice after being fed a WTD for 4 weeks contains a combination of both IFNγ and IL-4 cytokines as compared to chow serum (**B** and **C**). Bone marrow derived mast cells repeatedly incubated with fluid isolated from the PC-cavity of mice under 4 weeks of WTD increase their MHC-II expression as compared to chow PC-fluid (**D**). IFNγ and IL-4 cytokines are present in the peritoneal cell fluid of LDLr$^{-/-}$ mice fed 4 weeks of WTD (**E** and **F**). All values are shown as mean±SEM; ****p<0.001.*

Chapter 5

Disruption of a CD1d-mediated interaction between mast cells and NKT cells aggravates atherosclerosis

Manuscript submitted for publication

[1]Eva Kritikou
[1]Janine van Duijn
[1]Joya Nahon
[1]Thomas van der Heijden
[1]Merel Bouwman
[1]Christianne Groeneveldt
[1]Frank H. Schaftenaar
[1]Mara Kröner
[1]Johan Kuiper
[1]Gijs H.M. van Puijvelde
[1]Ilze Bot

[1] Division of Biopharmaceutics, Leiden Academic Centre for Drug Research, Leiden University, Leiden, The Netherlands

Abstract

The development of atherosclerosis is tightly regulated by the innate and adaptive immune system. Communication between these two compartments occurs, among others, also through lipid-antigen presentation by CD1d, a protein found on antigen presenting cells, to the NKT cell population. Recent evidence states that also mast cells express CD1d and directly communicate with NKT cells. However, no such relationship has been reported in atherosclerosis. Here, we aimed to elucidate the CD1d-mediated interaction between mast cells and NKT cells upon atherosclerosis progression *in vivo*. We adoptively transferred $CD1d^{-/-}$ or control mast cells to mast cell deficient $apoE^{-/-}/Kit^{W\text{-}sh/W\text{-}sh}$ mice and subsequently placed these animals on a Western-type diet for 10 weeks. In the course of the study, $CD1d^{-/-}$ mast cell restoration resulted in a mild increase in total serum cholesterol levels. At the endpoint, the aortic root of the $CD1d^{-/-}$ mast cell reconstituted mice showed increased plaque size with less collagen deposition and more intimal $CD4^{+}$ T cells, as compared to control mice. The T cell population in the spleen and circulation had higher T-bet expression and elevated pro-inflammatory cytokine production inside the aortic arch, in the form of IFNγ, TNFα and IL-17. This study is the first to illustrate that disruption of the CD1d communication pathway between mast cells and NKT cells aggravates atherosclerosis through a shift towards inflammatory T cell responses. This newly found ability of mast cell action during plaque progression sheds new light on their role in atherosclerosis.

1. Introduction

The accumulation and modification of lipids within large arteries is the essential step for the onset of atherosclerosis[1]. Yet, it is the activation of the innate and adaptive immune system that establishes the disease development, in the form of macrophage foam cells taking up these modified lipids and forming an atherosclerotic plaque[2]. Along with foam cell formation, macrophages process and present digested lipoprotein antigens to various T cell populations, through their MHC-machinery[3]. However, lipid fragments can also be presented through an MHC-I like protein named CD1d[4]. Presentation through CD1d is designed to specifically target and activate NKT cells, an adaptive cell population with unique T cell receptor (TCR) chains, tailored for endogenous and exogenous lipids[5]. In addition, these cells carry various markers of the innate NK cell population, therefore being the bridge between innate and adaptive immunity. Upon activation, NKT cells secrete vast amounts of T_{H1} and T_{H2} cytokines, such as IFNγ, TNFα or IL-4 and IL-13, respectively, depending on the quality and prevalence of their activating ligand[6,7]. Furthermore, their activation can be differentially shaped by co-stimulatory molecules, such as CD40L[8] and OX40[9]. However, it can also be cytokine-induced, independently of CD1d mediation[10,11]. NKT cells reside at high numbers in the liver[12], but have also been reported inside the atherosclerotic plaques of experimental models[13] and human atheromata[14], where they are thought to act in a proatherogenic manner[15]. NKT cells appear crucial mostly in the initial phase of the disease where they exacerbate plaque progression[13], until the adaptive response develops, whereupon primarily IFNγ producing $CD4^+$ T_{H1} cells[16] take over. However, NKT cells have also been described to influence advanced atherosclerosis, mainly by inducing plaque destabilization and necrotic core formation[17]. Previous studies have established that NKT cell activation through its most potent known activator, α-galactosylceramide (α-GalCer), increases atherosclerotic plaque progression in $apoE^{-/-}$ mice[17,18], whereas CD1d deficiency impairs neointima formation[19]. In addition, CD1d protein expression was elevated inside human atherosclerotic tissues in comparison to normal intima[20] and its role has been further confirmed in $CD1d^{-/-}LDLr^{-/-}$ mice, which upon high fat diet develop smaller plaques[17]. In fact, $CD1d^{-/-}$ mice completely lack NKT cells, indicating how essential CD1d molecules are for the thymic development of the NKT cell population[21]. NKT cells undergo maturation in the thymus and upon reaching stage 2, can migrate to the periphery[22]. Subsequently, these cells, whether in the periphery or in the thymus, may advance to stage 3 after glycolipid presentation through CD1d[23].

CD1d is expressed in various immune cells aside from macrophages. Professional antigen presenting cells such as dendritic cells and B cells are known activators of NKT cells through this receptor[24,25]. CD1d has also been reported to appear on the membrane of non-antigen presenting cell populations, such as the mast cells[26]. A recent

study suggested that CD1d expression on peritoneal mast cells can lead to NKT cell proliferation and cytokine release *in vitro*[27]. An indication of how this interaction may shape the immune response *in vivo* is given in allergic asthma where the CD1d-mediated action of mast cells on NKT cells aggravates airway inflammation by leading to more IgE production[26], a pathway known to trigger mast cell activation[28]. Mast cells are classical pro-inflammatory intermediaries in atherosclerosis[29], detected at an activated state inside atherosclerotic plaques[30]. Under the influence of modified lipids, mast cells secrete cytokines exacerbating disease progression[31], a characteristic that they share with NKT cells.

However, no direct interaction between these two cell types has been described in atherosclerosis before. Therefore, we aimed to examine whether an established communication, mediated by CD1d, between mast cells and NKT cells would affect disease progression *in vivo*. We adoptively transferred CD1d$^{-/-}$ or control mast cells into mast cell deficient apoE$^{-/-}$/Kit$^{W\text{-}sh/W\text{-}sh}$ mice and examined the progression of atherosclerosis in hyperlipidemic conditions.

2. Materials & Methods

2.1 Cell culture

Bone marrow isolated from female control LDLr$^{-/-}$ or from CD1d$^{-/-}$/LDLr$^{-/-}$ mice was cultured for 4 weeks at 37°C and 5% CO_2, in RPMI 1640 medium containing 25mM HEPES (Lonza) supplemented with 10% fetal calf serum (FCS), 60μM β-mercaptoethanol (Sigma), 100U/mL mix of penicillin/streptomycin (PAA), 1% non-essential amino-acids (NEAA; Gibco), 1% sodium pyruvate (Sigma) and 2% L-glutamine (Lonza) in the presence of cytokine IL-3 (5 ng/mL; Immunotools), to obtain mature mast cells. CD1d expression and mast cell purity, based on the expression of FcεRIα and CD117, were assessed at the adoptive transfer point (**Supplementary Figures 1A,B**).

2.2 Animals

All animal handling was executed in conformation with the guidelines of Directive 2010/63EU, as stated by the European Parliament and the experimental line was approved by the Leiden University Animal Ethics committee. Mice were bred and housed in the local facility with water and food supply *ad libitum*.

2.3 Atherosclerosis

Atherosclerosis-prone, mast cell deficient female apoE$^{-/-}$/Kit$^{W\text{-}sh/W\text{-}sh}$ mice with an average age of 15 weeks (n=10/grp) were adoptively transferred i.v. with $10*10^6$ fully maturated bone marrow derived mast cells, isolated from either LDLr$^{-/-}$ or CD1d$^{-/-}$LDLr$^{-/-}$ mice, hereafter referred to as control or CD1d$^{-/-}$ mast cells, respectively. After 4 weeks, mice were placed on a Western-Type diet (WTD) containing 0.25% cholesterol and 15% cocoa butter (SDS, Essex, UK) for a period of 10 weeks. The experimental set-up is described in **Supplementary Figure 1C**. During the study, tail blood was collected through the tail-vein, and serum was obtained upon

centrifugation at 8.000 rpm for 10 minutes. Total cholesterol levels were assessed for weeks 0, 3, 6 and 9 of the experiment, using an enzymatic colorimetric assay and compared to an internal Precipath control (Roche Diagnostics). Serum collected at the experimental endpoint was used for lipoprotein fraction separation (n=9 mice/grp). Samples were separated through a Superose 6 column (Smart Systems, Pharmacia) and the retrieved fractions were subsequently analysed for cholesterol levels. At the experimental end-point all animals were subcutaneous anesthetized with a mix of ketamine (40mg/mL), sedazine (6.25mg/mL) and atropine (0.05mg/mL).

2.4 Immunohistochemistry

The hearts of all mice were dissected below the atria and freshly frozen in OCT compound (Sakura). Hearts were sectioned, horizontally and towards the aortic arch. Upon identification of the trivalve leaflets of the aortic root, 10μm sections were collected. Mean plaque size was calculated for 7-8 sequential sections using an Oil-Red-O (ORO) staining (Sigma Aldrich). Macrophage content was assessed upon staining with a MOMA-2 antibody (1:1000, rat IgG2b, Serotec Ltd.). Intraplaque collagen was quantified using a Masson's Trichrome staining kit (Sigma Aldrich). The $CD4^+$ (1:90, clone RM4-5, BD Biosciences) and $CD8^+$ (1:100, clone Ly-2, BD Biosciences) T cell content in the aortic root was manually quantified. All morphometric analyses were performed in a blinded independent fashion on a Leica DM-RE microscope using a Leica QWin software (Leica Imaging Systems, UK).

2.5 Aortic arch cytokine stimulation

Aortic arches were digested in a mix of collagenase I (450u/mL; Sigma) and XI (250U/mL Sigma) and DNase (120U/mL; Sigma) and hyaluronidase (120 U/mL; Sigma). Single cells were obtained with a 70μm cell strainer. Subsequently cells were stimulated with a mix of PMA/Ionomycin (Sigma) in the presence of Brefeldin A (eBioscience) for 30 minutes. Cytokine production was measured using flow cytometry, after cell fixation and permeabilization (BD Bioscience).

2.6 Flow Cytometry

All blood and spleen samples were lysed with an erythrocyte lysis buffer (0.1mM EDTA, 10mM $NaHCO_3$, 1mM NH_4Cl, pH=7.2) to obtain white blood cells. Spleen and thymus samples were processed through a 70μm cell strainer for single cell selection. Single white blood (WB), thymus and spleen cells were stained with a viability dye solution as well as fluorescently labeled antibodies for extracellular proteins or permeabilized (Ebioscience) and subsequently stained with *intracellular* antibodies for transcription factor and cytokine determination (**Supplementary Table 1**). Measurements were performed on a FACS Canto II (Becton Dickinson, Mountain View, CA) and analysed using FlowJo software.

2.7 Statistics

All data are depicted as mean±SEM and analysed in GraphPad Prism 7. A 2-tailed Student *t*-test was used to compare between groups. Data were tested for normal distribution and Non-Gaussian distributed values were compared with a Mann-Whitney *U* test. Probability $P<0.05$ was considered significant.

3. Results

3.1 NKT cell levels are elevated upon CD1d$^{-/-}$ mast cell reconstitution

In this project, we aimed to examine the CD1d-mediated interaction between mast cells and NKT cells in atherosclerosis. We adoptively transferred CD1d$^{-/-}$ or control mast cells into mast cell deficient mice and subsequently fed both groups a WTD for 10 weeks. Upon 3 weeks of WTD, circulating CD1d-tetramer^{+} NKT cell levels were elevated in the CD1d$^{-/-}$ mast cell reconstituted group as compared to the control mast cell group (**Figure 1A.**, w3: CD1d$^{-/-}$: 3.04±0.5% vs control: 1.77±0.2%, P=0.0006). However, no difference was detected in the activated CD69^{+} circulating NKT cells (**Figure 1B**). At the experimental end-point the NKT cell population in the thymus was found increased upon CD1d$^{-/-}$ mast cell reconstitution, as compared to control mast cells (**Figure 1C**, CD1d$^{-/-}$: 0.65±0.05% vs control: 0.48±0.04%, P=0.014). Among the thymic NKT cells, particularly stage 2 CD24^{-}CD44^{+}NK1.1^{-} migrating NKT cells appeared elevated (**Figure 1D**, CD1d$^{-/-}$: 69.9±3.2% vs control: 59.2±2.8%, P=0.024) while stage 3, CD24^{-}CD44^{+}NK1.1^{+} NKT cells were slightly reduced in the CD1d$^{-/-}$ mast cell group, in comparison to control (**Figure 1E**, P=0.075). The liver NKT cell population did not differ between the groups (**Figure 1F**). However, the NKT cells of the CD1d$^{-/-}$ mast cell group were significantly less activated as compared to the control mast cell group (**Figure 1G**, CD1d$^{-/-}$: 55.9±1.8% vs control: 63.5±2.3%, P=0.018). It is worth noting that at the experimental endpoint we were able to detect a similar level of mast cells inside the peritoneal cavity in both groups of mice (**Supplementary Figure 2A**), whereas the CD40L levels on the NKT population of CD1d$^{-/-}$ reconstituted mice was found reduced as compared to control (**Supplementary Figure 2B**). The above findings indicate that lack of CD1d expression by the mast cells results in the migration of more stage 2 NKT cells from the thymus to the periphery, yet, in the liver these cells do not appear to get activated.

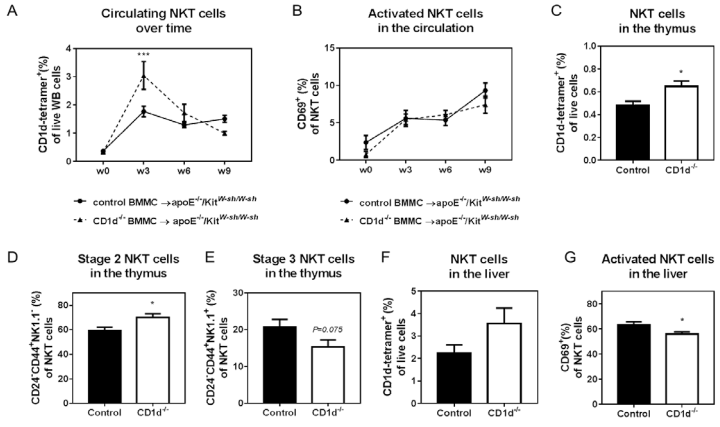


Figure 1: NKT cell levels are elevated but cells appear less active upon reconstitution of mast cell deficient mice with CD1d$^{-/-}$ mast cells. (**A**) Circulating levels of NKT cells increased at 3 weeks of WTD in the CD1d$^{-/-}$ reconstituted mice as compared to control. (**B**) No difference was detected on the NKT cell activation in the blood over time, between the groups. (**C**) Total thymic NKT cells as well as (**D**) migrating stage 2 NKT cells of mice carrying CD1d$^{-/-}$ mast cells were elevated, in comparison to NKT cells in mice reconstituted with control mast cells. (**E**) CD1d-dependent stage 3 NKT cells in the thymus, were slightly reduced in CD1d$^{-/-}$ reconstituted mice, as compared to control. (**F**) No difference in the total NKT cell populations of the liver between the two groups. (**G**) The NKT cells in the liver of CD1d$^{-/-}$ reconstituted mice showed decreased activation as compared to control mice. All values are depicted as mean±SEM. **P<0.05, ***P<0.001*

3.2 Serum cholesterol levels are elevated in the CD1d$^{-/-}$ mast cell reconstituted mice

In the course of the experiment, we analysed the total cholesterol levels in the serum of both groups of mice. We observed that after 3 weeks of WTD, total cholesterol was elevated in the circulation of CD1d$^{-/-}$ mast cell reconstituted mice, as compared to control mast cell repopulated mice (**Figure 2A**, w3: CD1d$^{-/-}$: 1258±74 mg/dL vs control: 1066±46 mg/dL, P=0.02). At the experimental endpoint, we further examined the lipoprotein fractions carried in the circulating cholesterol levels. We did not observe any difference among the (V)LDL and HDL fractions between the groups (**Figure 2B**).

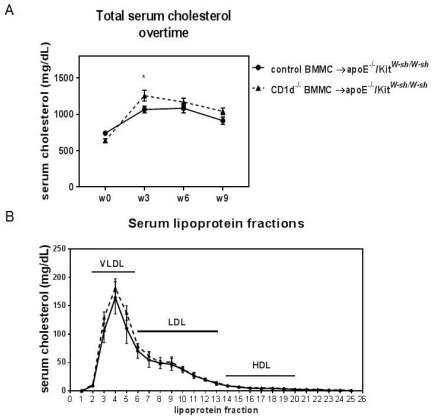


Figure 2: Cholesterol levels in the circulation show a mild increase in CD1d$^{-/-}$ mice at week 3. (**A**) Total cholesterol levels in the serum of CD1d$^{-/-}$ mast cell reconstituted mice showed a slight increase over time of WTD, as compared to control mast cell reconstituted mice. (**B**) No difference was observed in the lipoprotein fractions between the two groups of mice. **P<0.05*

3.3 CD1d-disruption on the mast cells increases atherosclerotic plaque size in the aortic root

After 10 weeks of WTD, the aortic root was analysed to determine atherosclerotic plaque size and morphology. Specifically, the atherosclerotic plaque size was analysed using an ORO staining (**Figure 3A**), the macrophage content in the area was detected using a MOMA-2 staining (**Figure 3B**) while the collagen composition of the plaque was established using a Masson's trichrome staining (**Figure 3C**). The atherosclerotic

plaque of the aortic root in CD1d$^{-/-}$ mast cell reconstituted mice was increased by 15% as compared to the plaque size of control mast cell reconstituted mice (**Figure 3D**, CD1d$^{-/-}$: 95*10^4±3.7*10^4 μm^2 vs control: 83*10^4±3.8*10^4 μm^2, P=0.033). Of note, the plaque development was elevated across the entire aortic root of the heart, measured from the start of the three-valve area up to the receding of the valves (**Supplementary Figure 3A**). Furthermore, the macrophage deposition in the plaque area of these mice was slightly elevated (**Figure 3E**, CD1d$^{-/-}$: 37*10^4±1.4*10^4 μm^2 vs control: 32*10^4±2.5*10^4 μm^2, P=0.066). In contrast, the plaques of the CD1d$^{-/-}$ mast cell reconstituted group showed reduced collagen content, as compared to control (**Figure 3F**, CD1d$^{-/-}$: 55.7±2% vs control: 61.1±1%, P=0.038).

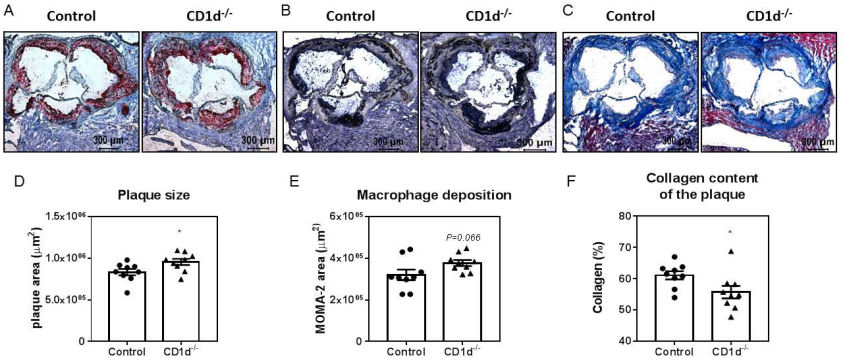


Figure 3: Mice reconstituted with CD1d$^{-/-}$ mast cells develop increased atherosclerosis in the aortic root, as compared to mice reconstituted with control mast cells. Representative pictures per group of mice for (**A**) ORO staining, (**B**) MOMA-2 staining and (**C**) Masson's trichrome staining (n=9/grp). The aortic root of mice reconstituted with CD1d$^{-/-}$ mast cells show (**D**) higher atherosclerotic plaques, (**E**) higher macrophage area and (**F**) lower collagen deposition, as compared to mice reconstituted with control mast cells. All values are depicted as mean±SEM. **P<0.05*

3.4 T cells within the atherosclerotic site produce pro-inflammatory cytokines

Upon further characterization of the aortic root area, we manually quantified the CD4$^+$ and CD8$^+$ T cell content. The number of CD4$^+$ T cells in the CD1d$^{-/-}$ mast cell mice showed a slight increase as compared to control (**Figure 4A**, CD1d$^{-/-}$: 14.9±4.2 cells/μm^2 vs control: 5.6±1.3 cells/μm^2, P=0.060). No difference was observed in the intimal CD8$^+$ T cells between the groups (**Figure 4B**). In addition, we analysed, using flow cytometry, the T cell content –as defined by Thy1.2$^+$ expression- in the aortic arch of these mice. The CD1d$^{-/-}$ mast cell bearing mice showed a mild reduction in the percentage of T cells in the area, in relation to control mice (**Figure 4C**, P=0.060). This was mainly attributed to a slight reduction in the CD8$^+$ T cell percentage (**Supplementary Figure 3B**). Therefore, the total T cell population present in the aortic arch of CD1d$^{-/-}$ mast cell reconstituted mice is characterized mainly by CD4$^+$ T cells (**Figure 4D**, CD1d$^{-/-}$: 32.2±2.8% vs control:

24.6±2.1%, P=0.041). Importantly, the T cells present in the aortic arch of these mice showed a substantial increase in pro-inflammatory cytokine production. Specifically, the aortic T cells of the CD1d$^{-/-}$ reconstituted group presented a significant increase in the production of cytokines IFNγ (**Figure 4E**, P=0.012), IL-17 (**Figure 4F**, P=0.025) and TNFα (P=0.0016) as opposed to the control group.

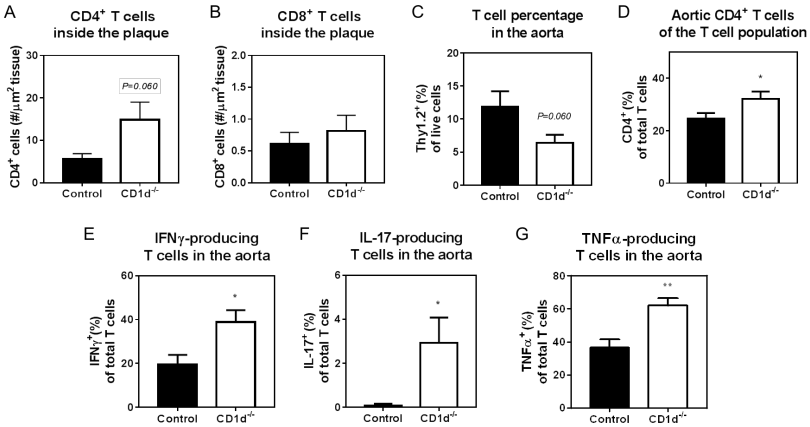


Figure 4: CD4$^+$ T cells are increased inside atherosclerotic sites of CD1d$^{-/-}$ mast cell reconstituted mice and produce higher amounts of pro-inflammatory cytokines. (**A**) CD4$^+$ T cells in the plaque intima of the aortic root appeared increased upon CD1d$^{-/-}$ mast cell reconstitution, as compared to control. (**B**) No difference was observed in the intimal CD8$^+$ T cell numbers between the groups. (**C**) Aortic T cells appear reduced in the aorta of the CD1d$^{-/-}$ reconstituted group as compared to control reconstituted mice. (**D**) The CD4$^+$ T cell levels of the total T cell population are elevated in the CD1d$^{-/-}$ mast cell group as compared to control. (**E**) IFNγ-producing, (**F**) IL-17-producing and (**G**) TNFα-producing T cells in the aorta of CD1d$^{-/-}$reconstituted mice are increased, in comparison to control mice. All values are depicted as mean±SEM. **P<0.05, **P<0.01*

3.5 T-bet expression is higher in the CD4$^+$ and CD8$^+$ T cell populations

Having in mind the close relation between NKT and T_{H1} cells, and upon observing the T cell responses in the atherosclerotic tissue, we aimed to analyse the systemic T cell response. We examined circulating and splenic T cells for the expression of transcription factor T-bet, which controls the secretion of pro-inflammatory cytokine IFNγ and CD4 T_{H1} cells [32]. In the blood of the CD1d$^{-/-}$ mast cell reconstituted mice we detected a substantial increase in T-bet expressing CD8$^+$ T cells, as compared to control (**Figure 5A**, CD1d$^{-/-}$: 8.2±0.9% vs control: 4.7±0.5%, P=0.004). Furthermore, while there was no difference in the percentage of T_{H1} cells (**Figure 5B**), the T-bet expression levels of the total CD4$^+$ T cell population were elevated in the CD1d$^{-/-}$ mast cell group in relation to control (**Figure 5C**, P=0.034). On the same line, T-bet expressing CD8$^+$ T cells were mildly elevated in the spleen of the CD1d$^{-/-}$ group (**Figure 5D**, P=0.077). The CD1d$^{-/-}$ mast cell carrying mice showed higher T_{H1} levels in their spleen, in relation

to the control group (**Figure 5E**, P=0.010). No difference was detected between the groups in the levels of T-bet expression within the entire CD4$^+$ T cell population of the spleen (**Figure 5F**). Of note, both groups showed a similar percentage of total CD8$^+$ and CD4$^+$ T cells in the blood (**Supplemental Figure 3C**,**D**) and spleen (**Supplemental Figure 3E**,**F**).

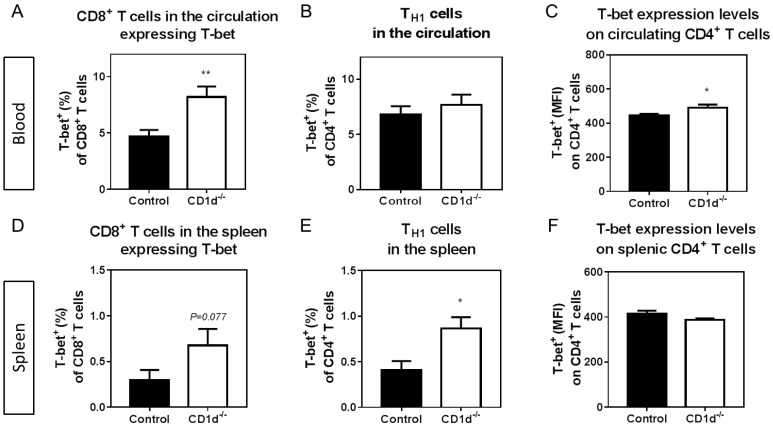


Figure 5: T-bet expression is increased in the circulating and splenic T cells of CD1d$^{-/-}$ mast cell reconstituted mice. (**A**) The percentage of T-bet expressing CD8$^+$ T cells in the blood was elevated upon CD1d$^{-/-}$ mast cell reconstitution as compared to control mast cell reconstitution (n=9/grp). (**B**) No difference was observed in the circulating CD4$^+$ T_{H1} cells between the groups; however (**C**) T-bet expressing CD4$^+$ T cells were elevated in the blood of CD1d$^{-/-}$ reconstituted mice as compared to control reconstituted mice. (**D**) Splenic T-bet expressing CD8$^+$ T cells (n=9/grp) as well as (**E**) CD4$^+$ T_{H1} cells are elevated in the spleen of CD1d$^{-/-}$ reconstituted mice in comparison to control reconstitution. (**F**) No difference was detected in the T-bet expression levels of the total CD4$^+$ T cell population between the two groups. All values are depicted as mean±SEM. **P<0.05, **P<0.01*

4. Discussion

In this study, we examined the CD1d-mediated effect of mast cells on NKT cells during atherosclerosis progression. We discovered that disruption of this axis aggravates atherosclerotic plaque development and destabilization through increased infiltration of CD4$^+$ T cells in the plaque intima. The T cell population in the plaques of mice which lacked CD1d-expressing mast cells showed high pro-inflammatory cytokine production, in the form of IFNγ, TNFα and IL-17. This may have caused the increased macrophage accumulation in the plaque. Furthermore, after 3 weeks of WTD, these animals showed a slight increase in serum cholesterol levels, and although temporary, it might have contributed to the observed plaque progression. The above results suggest that mast cell CD1d expression, influences the function of NKT cells and the subsequent control of CD4$^+$ T cell infiltration in a protective manner, which is previously unreported.

Early through WTD, following the adoptive transfer of CD1d$^{-/-}$ mast cells, we

detected an increase in the circulating levels of NKT cells. This increase was also apparent in the thymus of CD1d$^{-/-}$ mast cell reconstituted mice and could be explained by the elevated stage 2 NKT cells. As mentioned, thymic NKT cells of this stage migrate into peripheral tissues[33], which may thus explain their enhanced abundance in the circulation. However, while stage 2 are migrators and potent cytokine secretors, transition to stage 3 requires glycolipid presentation through CD1d[34]. Interestingly, we observed a reduction in the stage 3 thymic population of NKT cells, indicating that signal through CD1d is partially absent in the CD1d$^{-/-}$ mast cell reconstituted mice, as it was expected. This is implied also by the reduced activation and co-stimulation of the NKT population in the liver. These data suggest that the proatherogenic phenotype observed does not arise from a possible positive feedback signaling of other CD1d-expressing cells on the NKT population. It rather seems that NKT cells, generated in the thymus progress to stage 2 and migrate to the periphery at higher frequencies, perhaps to counteract for the absence in the CD1d signaling cascade initiated by the mast cells.

As stated before, NKT cells can potently activate T cells[35] and, in atherosclerosis, NKT cells are suggested to act prior to and pave the way for T cell infiltration in the plaque. In contrast to the existing concept on the proatherogenic role of NKT cells in atherosclerosis, and despite the reduced NKT cell activation, in this study we observed increased CD4$^+$ T cells within the plaque intima of the aortic root and an elevated cytokine production mainly by CD4$^+$ T cells of the aortic arch. While this seems surprising, particularly since CD1d signaling is known to induce a proatherogenic response on the NKT cells[18], a previously published study demonstrated that NKT cells in mice fed a high-fat diet, control T_{H1} cell responses specifically *via* a reduced IFNγ secretion in the serum upon α-GalCer injection[36]. Other reports have also stated that CD1d-mediated effects on the NKT cells can subsequently control T_{H1} cells. For example, in arthritis an autoimmune condition characterized by the presence of mast cells, loss of CD1d-mediated NKT activation exacerbated the T_{H1} responses, again in an IFNγ mediated manner[37]. In our study the pro-inflammatory IFNγ production of both the CD4$^+$ and CD8$^+$ T cell subsets seemed to be under the control of transcription factor T-bet, which showed elevated expression in T cells of both the spleen and the circulation. T-bet directly induces the production of IFNγ[38], whereas T_{H1} cells can also secrete TNFα[39]. In addition, NKT cells have been previously reported to inhibit T_{H17} cell differentiation[40], the main CD4$^+$ T cell subset secreting cytokine IL-17[41] that increases in proatherogenic conditions[42]. Therefore, it seems that loss of CD1d expression by the mast cells, and subsequent partial loss of NKT cell activation, led to an increase in T_{H1} cells, which in turn augmented atherosclerotic plaque progression. Hence, there is a tight balance between NKT and T_{H1} cells with mast cells playing a fundamental role in it.

The increase in pro-inflammatory CD4$^+$ T cells observed within the atherosclerotic

plaques of CD1d$^{-/-}$ mast cell reconstituted mice may have influenced the collagen deposition in the area. Specifically, the atherosclerotic plaques of these mice contained less collagen, signifying reduced plaque stability[43]. Cytokine IFNγ, has been accounted for its destabilizing manner[43,44]. Pro-inflammatory cytokine secretion by T cells could also explain the enhanced macrophage infiltration observed in the plaque, particularly since these cells inflict damage on the endothelium[45] and induce macrophage accumulation in the area[46]. Macrophages in turn produce a vast array of matrix metalloproteinases and are greatly implicated in plaque destabilization[47]. Therefore the increased macrophage content may have contributed to the destabilizing phenotype observed. It is also important to mention that the slight increase detected in circulating cholesterol levels may have contributed to the enhanced plaque development.

Up to date there are limited reports on the CD1d mediated action of mast cells on NKT cells. Mast cells on their own are potent cytokine secretors in organs where NKT cells reside, such as the thymus[48], the atherosclerotic plaque[29] and the liver[49]. Mast cell activation results in the release of proteases and cytokines, with IFNγ and TNFα considered among the typical cytokines excreted in their microenvironment. Furthermore, CD1d expression on the mast cell surface has been associated with mast cell activation *via* their classical receptor, FcεR[26]. Therefore, CD1d on the mast cell surface could also partly affect the activation of mast cells and, through that, the local response inside the plaque. The above study examining the function of mast cells and NKT cells, indicated that mast cells express also the costimulatory protein CD40; albeit not in a manner that influenced the NKT cell response in their experimental setup. Our results however showed that CD1d$^{-/-}$ mast cells seem to negatively affect the CD40L expression on liver NKT cells, thus supporting this mode of action. Additional co-stimulatory pathways may also participate in the observed mast cell-NKT cell interaction since ox40L and CD48 expression on the mast cells are reported to regulate cytokine secretion by NKT cells[27] .

To conclude, here we describe for the first time an *in vivo* interaction through mast cell-CD1d and NKT cells in atherosclerosis. This relationship seems to be protective, since its interruption increases atherosclerotic plaques, showing a highly pro-inflammatory CD4^{+} T cell content. A reduction in the mast cell-mediated CD1d activation of the NKT cells may therefore result in a compensatory operation of their close relatives, the T cells. While we have no further evidence of the exact lipid antigen presented by mast cell-CD1d to NKT cells, or why disruption of a classical proatherogenic pathway would enhance instead of hamper atherosclerosis, it is important to remember that NKT cells are also involved in many tolerogenic responses with presentation of endogenous lipids as to control autoimmune reactions[50]. Additionally, in the past it was shown that activation of NKT cells protects against autoimmune conditions, such as

diabetes[51] or arthritis[52]. In atherosclerosis, mast cells *via* the expression of CD1d and subsequent lipid presentation, appear to be fine-tuning the NKT cell actions, leading thus to a newly described mechanism of action in this disease.

Reference list:

1. Nakashima, Y., Wight, T. N. & Sueishi, K. Early atherosclerosis in humans: role of diffuse intimal thickening and extracellular matrix proteoglycans. *Cardiovasc. Res.* **79,** 14–23 (2008).
2. Legein, B., Temmerman, L., Biessen, E. A. L. & Lutgens, E. Inflammation and immune system interactions in atherosclerosis. *Cell. Mol. Life Sci.* **70,** 3847–3869 (2013).
3. Wolf, D., Zirlik, A. & Ley, K. Beyond vascular inflammation--recent advances in understanding atherosclerosis. *Cell. Mol. Life Sci.* **72,** 3853–3869 (2015).
4. Bondarenko, S., Catapano, A. L. & Norata, G. D. The CD1d-natural killer T cell axis in atherosclerosis. *J. Innate Immun.* **6,** 3–12 (2014).
5. Skold, M., Faizunnessa, N. N., Wang, C. R. & Cardell, S. CD1d-specific NK1.1+ T cells with a transgenic variant TCR. *J. Immunol.* **165,** 168–174 (2000).
6. Qiao, X. *et al.* OCH-mediated shift of Th1 and Th2 cytokines by NKT cells in mice with aplastic anemia. *Med. Oncol.* **32,** 67 (2015).
7. East, J. E., Kennedy, A. J. & Webb, T. J. Raising the roof: the preferential pharmacological stimulation of Th1 and th2 responses mediated by NKT cells. *Med. Res. Rev.* **34,** 45–76 (2014).
8. Oki, S., Tomi, C., Yamamura, T. & Miyake, S. Preferential T(h)2 polarization by OCH is supported by incompetent NKT cell induction of CD40L and following production of inflammatory cytokines by bystander cells in vivo. *Int. Immunol.* **17,** 1619–1629 (2005).
9. Zhou, D. OX40 signaling directly triggers the antitumor effects of NKT cells. *J. Clin. Invest.* **117,** 3169–3172 (2007).
10. Leite-De-Moraes, M. C. *et al.* A distinct IL-18-induced pathway to fully activate NK T lymphocytes independently from TCR engagement. *J. Immunol.* **163,** 5871–5876 (1999).
11. Bourgeois, E. *et al.* The pro-Th2 cytokine IL-33 directly interacts with invariant NKT and NK cells to induce IFN-gamma production. *Eur. J. Immunol.* **39,** 1046–1055 (2009).
12. Major, A. S. *et al.* Quantitative and qualitative differences in proatherogenic NKT cells in apolipoprotein E-deficient mice. *Arterioscler. Thromb. Vasc. Biol.* **24,** 2351–2357 (2004).
13. Aslanian, A. M., Chapman, H. A. & Charo, I. F. Transient role for CD1d-restricted natural killer T cells in the formation of atherosclerotic lesions. *Arterioscler. Thromb. Vasc. Biol.* **25,** 628–632 (2005).
14. Bobryshev, Y. V & Lord, R. S. A. Co-accumulation of dendritic cells and natural killer T cells within rupture-prone regions in human atherosclerotic plaques. *J. Histochem. Cytochem.* **53,** 781–785 (2005).
15. van Puijvelde, G. H. M. & Kuiper, J. NKT cells in cardiovascular diseases. *Eur. J. Pharmacol.* (2017). doi:10.1016/j.ejphar.2017.03.052
16. Frostegard, J. *et al.* Cytokine expression in advanced human atherosclerotic plaques: dominance of pro-inflammatory (Th1) and macrophage-stimulating cytokines. *Atherosclerosis* **145,** 33–43 (1999).
17. Nakai, Y. *et al.* Natural killer T cells accelerate atherogenesis in mice. *Blood* **104,** 2051–2059 (2004).
18. Tupin, E. *et al.* CD1d-dependent activation of NKT cells aggravates atherosclerosis. *J. Exp. Med.* **199,** 417–422 (2004).
19. Strom, A. *et al.* Involvement of the CD1d-natural killer T cell pathway in neointima formation after vascular injury. *Circ. Res.* **101,** e83-9 (2007).
20. Melian, A., Geng, Y. J., Sukhova, G. K., Libby, P. & Porcelli, S. A. CD1 expression in human atherosclerosis. A potential mechanism for T cell activation by foam cells. *Am. J. Pathol.* **155,** 775–786 (1999).

21. Gapin, L., Matsuda, J. L., Surh, C. D. & Kronenberg, M. NKT cells derive from double-positive thymocytes that are positively selected by CD1d. *Nat. Immunol.* **2,** 971–978 (2001).
22. Das, R., Sant'Angelo, D. B. & Nichols, K. E. Transcriptional control of invariant NKT cell development. *Immunol. Rev.* **238,** 195–215 (2010).
23. Godfrey, D. I., Stankovic, S. & Baxter, A. G. Raising the NKT cell family. *Nat. Immunol.* **11,** 197–206 (2010).
24. Keller, C. W., Freigang, S. & Lunemann, J. D. Reciprocal Crosstalk between Dendritic Cells and Natural Killer T Cells: Mechanisms and Therapeutic Potential. *Front. Immunol.* **8,** 570 (2017).
25. Chaudhry, M. S. & Karadimitris, A. Role and regulation of CD1d in normal and pathological B cells. *J. Immunol.* **193,** 4761–4768 (2014).
26. Hong, G. U., Kim, N. G., Kim, T. J. & Ro, J. Y. CD1d expressed in mast cell surface enhances IgE production in B cells by up-regulating CD40L expression and mediator release in allergic asthma in mice. *Cell. Signal.* **26,** 1105–1117 (2014).
27. Gonzalez Roldan, N., Orinska, Z., Ewers, H. & Bulfone-Paus, S. CD252 regulates mast cell mediated, CD1d-restricted NKT-cell activation in mice. *Eur. J. Immunol.* **46,** 432–439 (2016).
28. Galli, S. J. & Tsai, M. IgE and mast cells in allergic disease. *Nat. Med.* **18,** 693–704 (2012).
29. Bot, I., Shi, G.-P. & Kovanen, P. T. Mast cells as effectors in atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* **35,** 265–271 (2015).
30. Kaartinen, M., Penttila, a. & Kovanen, P. T. Accumulation of activated mast cells in the shoulder region of human coronary atheroma, the predilection site of atheromatous rupture. *Circulation* **90,** 1669–1678 (1994).
31. Lappalainen, J., Lindstedt, K. A., Oksjoki, R. & Kovanen, P. T. OxLDL-IgG immune complexes induce expression and secretion of proatherogenic cytokines by cultured human mast cells. *Atherosclerosis* **214,** 357–363 (2011).
32. Miller, S. A. & Weinmann, A. S. Molecular mechanisms by which T-bet regulates T-helper cell commitment. *Immunol. Rev.* **238,** 233–246 (2010).
33. Matsuda, J. L. *et al.* T-bet concomitantly controls migration, survival, and effector functions during the development of Valpha14i NKT cells. *Blood* **107,** 2797–2805 (2006).
34. Gumperz, J. E., Miyake, S., Yamamura, T. & Brenner, M. B. Functionally distinct subsets of CD1d-restricted natural killer T cells revealed by CD1d tetramer staining. *J. Exp. Med.* **195,** 625–636 (2002).
35. Nishimura, T. *et al.* The interface between innate and acquired immunity: glycolipid antigen presentation by CD1d-expressing dendritic cells to NKT cells induces the differentiation of antigen-specific cytotoxic T lymphocytes. *Int. Immunol.* **12,** 987–994 (2000).
36. Miyazaki, Y. *et al.* Effect of high fat diet on NKT cell function and NKT cell-mediated regulation of Th1 responses. *Scand. J. Immunol.* **67,** 230–237 (2008).
37. Teige, A. *et al.* CD1d-dependent NKT cells play a protective role in acute and chronic arthritis models by ameliorating antigen-specific Th1 responses. *J. Immunol.* **185,** 345–56 (2010).
38. Lugo-Villarino, G., Maldonado-Lopez, R., Possemato, R., Penaranda, C. & Glimcher, L. H. T-bet is required for optimal production of IFN-gamma and antigen-specific T cell activation by dendritic cells. *Proc. Natl. Acad. Sci. U. S. A.* **100,** 7749–7754 (2003).
39. Cantor, J. & Haskins, K. Effector function of diabetogenic CD4 Th1 T cell clones: a central role for TNF-alpha. *J. Immunol.* **175,** 7738–7745 (2005).
40. Oh, K., Byoun, O.-J., Ham, D.-I., Kim, Y. S. & Lee, D.-S. Invariant NKT cells regulate experimental autoimmune uveitis through inhibition of Th17 differentiation. *Eur. J. Immunol.* **41,** 392–402 (2011).

41. Gaffen, S. L. Structure and signalling in the IL-17 receptor family. *Nat. Rev. Immunol.* **9,** 556–567 (2009).

42. Lim, H. *et al.* Proatherogenic conditions promote autoimmune T helper 17 cell responses in vivo. *Immunity* **40,** 153–165 (2014).

43. Halvorsen, B. *et al.* Atherosclerotic plaque stability--what determines the fate of a plaque? *Prog. Cardiovasc. Dis.* **51,** 183–194 (2008).

44. Amento, E. P., Ehsani, N., Palmer, H. & Libby, P. Cytokines and growth factors positively and negatively regulate interstitial collagen gene expression in human vascular smooth muscle cells. *Arterioscler. Thromb. a J. Vasc. Biol.* **11,** 1223–1230 (1991).

45. Nakajima, T. *et al.* T-cell-mediated lysis of endothelial cells in acute coronary syndromes. *Circulation* **105,** 570–575 (2002).

46. Galkina, E. *et al.* CXCR6 promotes atherosclerosis by supporting T-cell homing, interferon-gamma production, and macrophage accumulation in the aortic wall. *Circulation* **116,** 1801–1811 (2007).

47. Newby, A. C. Metalloproteinases promote plaque rupture and myocardial infarction: A persuasive concept waiting for clinical translation. *Matrix Biol.* **44–46,** 157–166 (2015).

48. Ribatti, D. & Crivellato, E. The role of mast cell in tissue morphogenesis. Thymus, duodenum, and mammary gland as examples. *Exp. Cell Res.* **341,** 105–109 (2016).

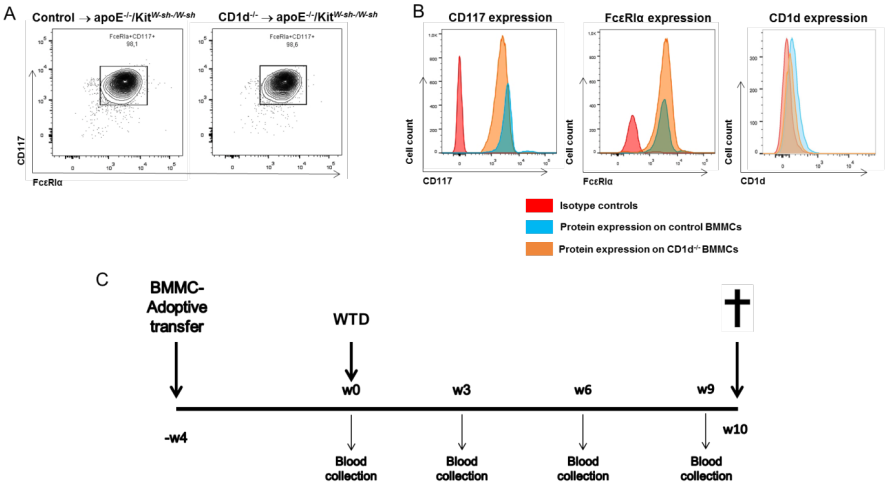
49. Jarido, V. *et al.* The emerging role of mast cells in liver disease. *Am. J. Physiol. Gastrointest. Liver Physiol.* ajpgi.00333.2016 (2017). doi:10.1152/ajpgi.00333.2016

50. Godfrey, D. I. & Kronenberg, M. Going both ways: immune regulation via CD1d-dependent NKT cells. *J. Clin. Invest.* **114,** 1379–1388 (2004).

51. Hong, S. *et al.* The natural killer T-cell ligand alpha-galactosylceramide prevents autoimmune diabetes in non-obese diabetic mice. *Nat. Med.* **7,** 1052–1056 (2001).

52. Coppieters, K. *et al.* A single early activation of invariant NK T cells confers long-term protection against collagen-induced arthritis in a ligand-specific manner. *J. Immunol.* **179,** 2300–2309 (2007).

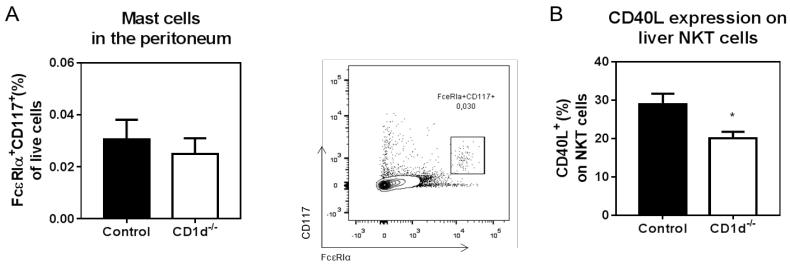
Supplementary Information



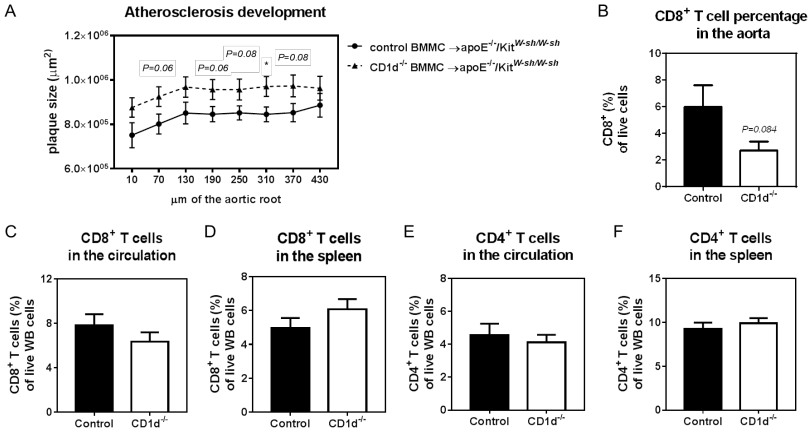
Supplemental Figure 1: Experimental set-up and controls. (**A**) Flow cytometry purity check, 1 day prior to mast cell reconstitution. (**B**) Isotype controls for CD117, FcεRIα and CD1d expression on the transferred BMMCs. (**C**) Mast cell deficient apoE/Kit$^{W\text{-}sh/W\text{-}sh}$ mice were adoptively transferred through i.v. injections with 10*10^6 mature bone marrow derived mast cells of either CD1d$^{-/-}$ mice or control control mice. After a 4 week-recovery period, the mice were placed on a WTD for an additional 10 weeks.

Antibody	Clone	Concentration	Fluorochrome	Company
Fixable Viability Dye	-	0.05 µg/sample	eFluor 780	eBioscience
FcεRIα	MAR-1	0.1 µg/sample	PercP Cy5.5	Biolegend
FcεRIα	MAR-1	0.1 µg/sample	APC	eBioscience
CD117	2B8	0.1 µg/sample	APC/PE Cy7	eBioscience
CD1d	1B1	0.1 µg/sample	PE	eBioscience
CD1d-tetramer	-	0.125 µg/sample	BV 420	-
CD69	H1.2F3	0.1 µg/sample	PE	eBioscience
CD24	M1/69	0.1 µg/sample	PercP Cy5.5	BD Biosciences
CD44	IM7	0.1 µg/sample	PE Cy7	eBioscience
NK1.1	PK136	0.1 µg/sample	FITC	eBioscience
CD40L	MR1	0.1 µg/sample	APC	eBioscience
CD4	GK1.5	0.1 µg/sample	FITC/eFluor 450	eBioscience
CD8a	53-6.7	0.1 µg/sample	PercP	BD Biosciences
CD8a	53-6.7	0.1 µg/sample	APC	eBioscience
Thy1.2	53-2.1	0.1 µg/sample	PE Cy7	eBioscience
IFNγ	XMG1.2	*0.3 µg/sample*	BV650	BD Biosciences
IL-17	eBio17B7	*0.3 µg/sample*	Alexa Fluor 488	eBioscience
TNFα	MP6-XT22	*0.3 µg/sample*	PE	eBioscience
T-bet	eBio4B10	*0.3 µg/sample*	Alexa Fluor 660	eBioscience

Supplementary Table 1. Flow cytometry antibodies: List of extracellular and *intracellular* antibodies



Supplemental Figure 2: Mast cells are detected in the peritoneum of mast cell deficient apoE$^{-/-}$/Kit$^{W\text{-}sh/W\text{-}sh}$ mice in both groups at the experimental endpoint. (A) Mast cell levels in the peritoneum of apoE$^{-/-}$/Kit$^{W\text{-}sh/W\text{-}sh}$ mice reconstituted with either control or CD1d$^{-/-}$ BMMCs show no difference between the two groups. (B) The percentage of liver NKT cells that express the costimulatory molecule CD40L is decreased upon CD1d$^{-/-}$ BMMC reconstitution, as compared to control BMMC reconstitution. All values are depicted as mean±SEM. **P<0.05*



Supplemental Figure 3: Atherosclerotic plaques in the heart of the CD1d$^{-/-}$ reconstituted mice develop at a higher rate across the aortic root area. (**A**) CD1d$^{-/-}$ mast cell reconstitution results in increased atherosclerosis development through the aortic root, as compared to reconstitution of mice with control mast cells (n=9/grp). (**B**) The CD8^{+} T cell content in the aortas of CD1d$^{-/-}$ reconstituted mice is slightly reduced in comparison to control mice. No difference was detected in the total (**C**) circulating CD8^{+} (**D**) splenic CD8^{+} or (**E**) circulating CD4^{+} T cell levels and (**F**) spleen CD4^{+} T cells between the two groups of mice. All values are depicted as mean±SEM. **P<0.05*

Chapter 6

Phenotypic characterization of human intraplaque mast cells using flow cytometry

Manuscript in preparation

[1]Eva Kritikou
[1]Bram Slütter
[1]Janine van Duijn
[1]Amanda C. Foks
[1]Thomas van der Heijden
[2]Anouk Wezel
[2]Harm J. Smeets
[1]Johan Kuiper
[1]Ilze Bot

[1] Division of Biopharmaceutics, Leiden Academic Centre for Drug Research, Leiden University, Leiden, The Netherlands

[2]Department of Surgery. HMC Westeinde, The Hague, The Netherlands

Abstract

Human mast cells have previously been associated with adverse cardiovascular events. Mast cell activation, through the classical antigen sensitized-IgE binding to their characteristic Fcε-receptor, causes the release of their cytoplasmic granules. These granules are filled with neutral proteases, such as chymase and tryptase, histamine and pro-inflammatory mediators. Mast cells accumulate at high numbers within the human atherosclerotic tissue, particularly in the shoulder region of the plaque. However, their activation mechanisms, as well as their protease content, locally in the plaque, are still largely unclear. This is the first study to use flow cytometry for the analysis of the mast cell content in 22 human plaque samples, collected after femoral and carotid endarterectomy surgery. We observed that most intraplaque mast cells are activated, based on their CD63 protein expression. Furthermore, we detected that most of the activated mast cells had IgE fragments bound on their surface, while a fraction showed IgE-independent activation. Finally, we confirm previous reports stating that, while the majority of mast cells contain both chymase and tryptase, there is remarkable heterogeneity in the distribution of these proteases within the mast cell population per individual. In conclusion, this project establishes the strong relation between the presence of IgE and the activation of mast cells, which leads to the subsequent secretion of their protease content inside atherosclerotic plaques. Our data pave the way for potential therapeutic intervention through targeting IgE-mediated actions in human atherosclerosis.

Report

Up to the present day, atherosclerosis, the main underlying pathology of acute cardiovascular syndromes like stroke or myocardial infarction, is the major cause of human mortality[1]. As in most pathological conditions, the response of the immune system is crucial in the advancement of atherosclerosis, with the mast cells being key mediators in this process[2]. Mast cells are innate immune cells, mainly characterized by their notorious granular load release, upon activation with antigen-sensitized IgE fragments in allergy[3]. Aside from allergic inflammation, mast cells have long been established to play an important pro-inflammatory role in the development of atherosclerosis, as detected by both experimental studies as well as in human subjects[4]. Mast cells reside at low numbers in normal arterial tissue, however they are found to accumulate in arteries where a lipid-rich atherosclerotic plaque is formed[5]. In fact, as human atherosclerosis progresses, mast cells become increasingly activated whereupon they excrete their granules in the surrounding tissue[6]. The degranulated material consists mainly of proinflammatory cytokines, histamine and neutral proteases, such as chymase and tryptase[7]. Mast cell activation is reported to augment plaque progression[8], enhance plaque destabilization[9] and increase the levels of intraplaque hemorrhage incidence[10]. Previous attempts to characterize mast cells and their protease content in human atheromata, by the means of immunohistochemistry, has revealed that they comprise a very heterogeneous population; the majority contain only tryptase, while a smaller but significant proportion contains chymase as well[11]. This heterogeneity is particularly interesting considering that diversity in the quality and quantity of the mast cell degranulating material may exert differential effects inside the atherosclerotic plaque. For example, while mast cell chymase seems to impede tissue repair after a myocardial infarction episode[12], tryptase has been reported to participate in the healing process[13]. Furthermore, mast cells inside human plaques have been found to correlate with typical atherogenic immune populations like dendritic cells and T cells[14]. Importantly, in a human study of 270 patients, intraplaque mast cells emerge as the primary immune cell type to positively associate with future cardiovascular events[15]. Until this day, the means by which mast cells get activated inside the atherosclerotic plaque have not been elucidated in full detail. However, it is suggested that the classical IgE-sensitized pathway[16,17] is involved up to a certain extent. Nevertheless, there is increasing evidence that additional atherosclerosis-specific mechanisms can trigger mast cell activation, independently of IgE-binding[18,19] such as through TLRs[20], complement[21] or neuropeptide[22] receptors. Thus far, the proportional effect of these distinct pathways involved in atherosclerosis, as well as the exact phenotype of mast cells inside human plaques, have not been clarified.

In this technical report of an ongoing cohort study, we made use of the flow

cytometry method to phenotypically characterize human intraplaque mast cells. The atherosclerotic plaque material of 22 human subjects was collected post-operatively from carotid and femoral arteries. Specifically, anonymous individuals, for whom we did not receive any patient details, underwent endarterectomy surgery in a period between July and December 2016 at the Haaglanden Medical Center (HMC), Westeinde, The Hague, NL, after which the atherosclerotic plaque was collected. The handling of all human samples complied with the "Code for Proper Secondary Use of Human Tissue". Human plaques were processed into single cell suspensions by a 2-hour digestion round at 37°C, with an enzyme mix consisting of collagenase IV (Gibco) and DNase (Sigma) as previously described[23]. Subsequently, the samples were filtered through a 70μm cell strainer to obtain single cells. The cells were further stained with extracellular antibodies containing a fluorescent label or fixated and permeabilized (BD Biosciences) for intracellular staining (**Table 1**). Fluorescently labeled samples were measured on a FACS Canto II (BD Biosciences) or Cytoflex (Beckman Coulter) and analyzed using FlowJo software. All data are depicted using GraphPad Prism 7. Values were tested for normalcy. Upon non-Gaussian distribution, an unpaired Mann Whitney *U*-test was performed. In the case of more than two groups a one way-ANOVA analysis was used. Differences lower than $P<0.05$ were considered statistically significant.

Antibody	Fluorochrome	Clone	Concentration	Company
Fixable Viability Dye	eFluor 780	-	0.1μg/sample	eBioscience
CD45	PB	2D1	0.25μg/sample	eBioscience
FcεRIα	APC/PE Cy7	AER-37	0.12 μg/sample	eBioscience
CD117	PercP Cy5.5	104D2	0.1μg/sample	eBioscience
CD63	PE	H5C6	0.1μg/sample	eBioscience
IgE	PE Cy7/FITC	Ige21	0.25μg/sample	eBioscience
Chymase/CMA1	*Alexa Fluor 647*	-	0.1μg/sample	Bioss Antibodies
Tryptase/TPSAB1	*PE*	-	0.1μg/sample	LSBio

Table 1: List of extracellular and *intracellular* antibodies used.

In **Figure 1A** we demonstrate the gating strategy that we followed to detect human intraplaque mast cells. Specifically, we pre-selected all cells from the debris present in the human plaques, based on their size (FSC) and granularity (SSC). Of these, single cells were further separated according to their width (FSC-W) and area (FSC-A). In addition, viability was detected according to the negative binding of a fluorescent viability dye (FVD^-). Viable white blood cells were separated based on the expression of the panleukocyte marker CD45. Of these cells, we were able to detect the population of intraplaque mast cells according to the high expression of their characteristic markers FcεRIα, the ligand for IgE[24] and of CD117, the receptor for stem cell factor, a growth factor that is required for the end-stage maturation of mast cells[25]. Accordingly, we observed

that the percentage of mast cells, out of all leukocytes present inside atherosclerotic plaques, is 1.19±0.31% for carotid arteries (n=9) and 1.32±0.21% for femoral arteries (n=13) (**Figure 1B**). Of note, a number of leukocytes may be contained in the excluded debris material. Nonetheless we enumerated the viable cells after manual quantification using both Trypan Blue and in relation to the percentage of viable, $CD45^+$ cells observed according to our gating strategy. We detected that carotid plaques consisted of a mean 12.386±4.961 mast cells while femoral plaques contained 94.273±37.420 mast cells. We were able to isolate more inflammatory cells from femoral as compared to carotid arteries, and thus we identified increased mast cell numbers in femoral plaques (P=0.081; **Figure 1C**). Indeed, femoral plaque samples overall tended to show higher total leukocyte content as compared to carotid plaques (carotid: $12*10^5$±$4.7*10^5$ leukocytes vs femoral:$76*10^5$±$27*10^5$ leukocytes, P=0.081; **Figure 1D**).

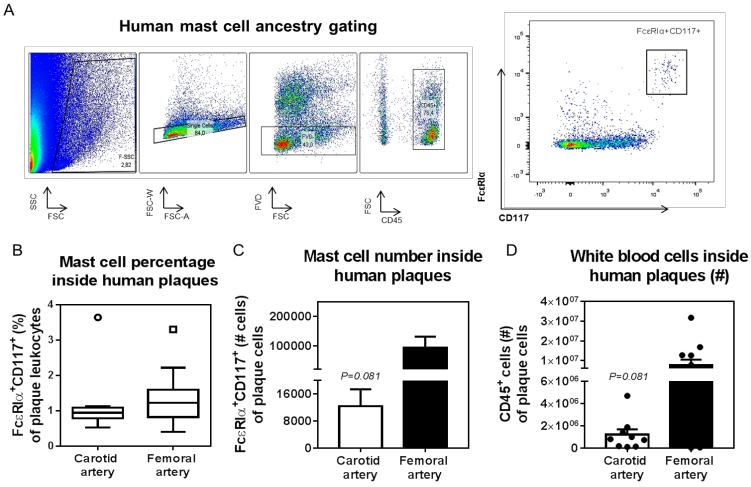


Figure 1: Mast cell content in human plaques. (**A**) Human mast cell gating strategy using flow cytometry. Human intraplaque cells were selected based on their size and area. Viable cells were further separated according to the negative incorporation of fluorescent viability dye (FVD^-). Immune cells were detected using the pan-leukocyte marker $CD45^+$ and the human mast cell population was further classified using antibodies against their characteristic markers $Fc\varepsilon RI\alpha^+$ and $CD117^+$. (**B**) Mast cell percentage inside human plaques isolated upon endarterectomy surgeries in carotid and femoral arteries. (**C**) Mast cell absolute numbers inside human carotid and femoral artery samples. The femoral arteries show a slight increase in mast cell content. (**D**) Femoral artery plaques show an increased number of white blood cells as compared to the carotid artery plaques, based on the expression of marker CD45. *Percentages are depicted in a Tukey box plot. Cell numbers are depicted as mean±SEM; (n=10-12/grp).*

We proceeded to characterize the status of the detected human intraplaque cells. Specifically, we stained them for the levels of IgE bound on their surface and observed that inside carotid arteries: 7.779±3.419 intraplaque mast cells, or approximately 63% of the total mast cell population, contained IgE, whereas in the femoral plaques

77.493±35.927 mast cells, or 82% of all mast cells had IgE on their surface (P=0.058; **Figure 2A**). Because IgE binding usually implies mast cell degranulation, we also screened our cells for the expression of CD63, a lysosomal protein that fuses with the membrane upon release of cellular content and therefore marks mast cell activation[26]. We detected 6.998±3.530 $CD63^+$ mast cells in carotid plaques, which indicates about 56% of these cells being in an activated state, while femoral plaques showed 57.417±23.152 activated mast cells, at a total percentage of 61% (**Figure 2B**). We were particularly interested to determine the proportion of IgE mediated activation per patient. For that reason, we analyzed within each arterial plaque sample the population of mast cells that showed IgE bound and also expressed CD63 (IgE^+CD63^+), as opposed to only IgE-binding (IgE^+CD63^-) and only CD63 expression (IgE^-CD63^+) (**Figure 2C**). We discovered that 23.81±3.7% of all human plaque mast cells have IgE bound on their surface without being activated, whereas the majority of mast cells, with 40.01±3.9%, appeared to have IgE bound on their surface and had also undergone degranulation. Interestingly, a proportion of mast cells, namely 19.6±2.9%, appeared to be activated without showing any IgE-fragments bound on their surface, suggesting that this mast cell fraction had been activated *via* alternative mast cell activation pathways. The IgE-activated population was, however, significantly higher than the cells that were subjected to non-IgE mediated activation (P=0.0005), but also higher than the mast cell population that showed binding of IgE without being activated (P=0.0067).

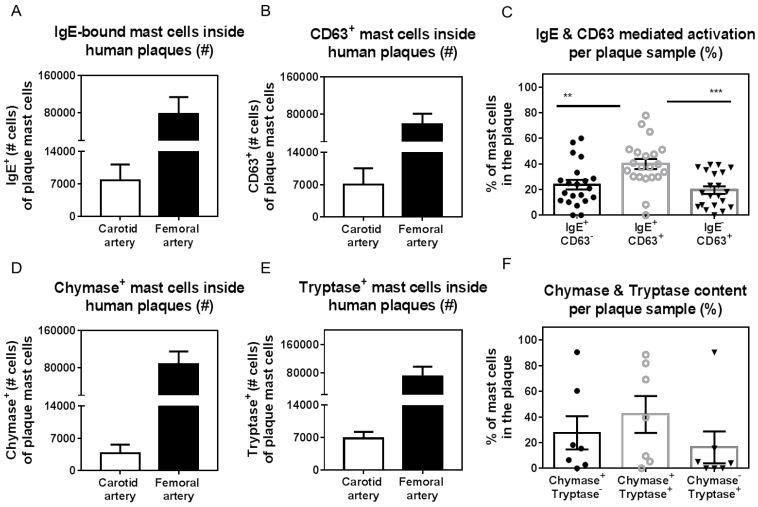


Figure 2: Basic characterization of human intraplaque mast cells. (**A**) IgE-antigen bound mast cells within the atherosclerotic plaques of human carotid and femoral arteries. (**B**) Number of activated mast cells, as defined by marker CD63, inside carotid and femoral artery human plaques. (**C**) Percentage of IgE-bound, IgE-activated and non-IgE activated mast cells per artery sample (n=22). (**D**) Chymase-containing mast cell numbers in human carotid and femoral plaques.(**E**) Number of mast cells containing tryptase in human carotid and femoral arteries. (**F**) Chymase-only, chymase and tryptase-containing and, tryptase-only mast cell percentages per artery sample (n=7). *All values are depicted as mean±SEM. **P<0.01, ***P<0.001*

In a smaller sample of the same patients (n=7) we quantified the mast cell populations that contained chymase and tryptase. Regarding chymase expression, we detected 3.769±1.847 mast cells (30%) inside carotid plaques, and 86.805±28.025 mast cells (92%) inside femoral plaques containing this protease (**Figure 2D**). On the other hand, 6.807±1.439 (54%) of carotid intraplaque mast cells, and 70.627±27.340 (75%) of femoral intraplaque mast cells contained tryptase (**Figure 2E**). Aiming to assess the distribution of chymase and tryptase contents within the mast cell population of the same patients, we analyzed the mast cells consisting of only chymase ($Chymase^{+}Tryptase^{-}$) or only tryptase ($Chymase^{-}Tryptase^{+}$), as compared to the mast cells containing both proteases ($Chymase^{+}Tryptase^{+}$) (**Figure 2F**). We observed that the majority of mast cells, namely 42.0±14.4% were positive for both proteases, while 27.7±13% contained only chymase and 16.4±12.5% only tryptase.

Overall, in this report we are the first to characterize intraplaque mast cells in human atherosclerosis. We provide evidence that the majority of mast cells present in arteries with advanced atherosclerosis are activated through IgE binding, while a smaller fraction can undergo non-IgE dependent activation. We also show that, as mentioned previously[11], human intraplaque mast cells mainly contain both chymase and tryptase, while we detected smaller percentages of either chymase-only, or tryptase-only carrying mast cells; thus confirming the concept of high heterogeneity in the protease content of mast cells in atherosclerotic disease.

Interestingly, while IgE levels in the circulation[17] have been linked to an increased incidence of acute cardiovascular events and IgE fragments have been previously reported inside human atheromatic tissue[16], up to now it was not clear to which extent this pathway affects the activation of mast cells in the area. Our data confirm that most mast cells present in atherosclerotic plaques are activated[5,10], as it has been shown previously specifically for the shoulder region. Yet, what we show here is that the main activating mechanism is through classical antigen-sensitized IgE binding on their surface Fcε-receptors. Nonetheless, this does not seem to be the only way by which mast cells are activated in human atherosclerosis. It is still unclear what this implies in terms of mediator release. It would be interesting to further characterize the intracellular content of these cells to examine if different activation pathways lead to the release of different proteases and cytokines, and how this may possibly affect the surrounding environment. In addition, we show that a small proportion of cells bind IgE without undergoing activation. Circulating IgE can thus bind on the surface of intraplaque mast cells and sensitize them prior to antigen binding. The exact antigenic fragment that may cause intraplaque mast cell activation has not yet been identified but binding of lipid specific antigenic fragments is the most plausible idea[20]. Furthermore, the detection of IgE fragments inside the plaque tissue confirms that these fragments

can surpass the endothelial wall barrier and accumulate in the plaque area, which may explain the reason why circulating IgE levels correlate with end-stage cardiovascular events like atherothrombosis[27] and myocardial infarction[28]. It is thus reasonable to acknowledge IgE as an important risk factor in cardiovascular episodes, even though it still remains to be elucidated whether it is a causative element[29]. In addition, our data raise an interesting question regarding patients who suffer from other syndromes with increased circulating IgE levels. The development of atherosclerosis is a chronic process, which spans from the formation of a fatty streak, during an individual's teenage years and may result in an unfortunate acute event of an end-stage plaque rupture and vessel occlusion[30]. In the course of those years a fraction of humans may be diagnosed with allergic inflammatory conditions, associating with high levels of circulating IgE[31]. This may mean that IgE has an increased chance to migrate through the endothelium and bind intraplaque mast cells, raising thus the likelihood for a future cardiovascular event. In fact, there is compiling evidence that allergic asthma and atherosclerosis are linked[32], and mast cells are seemingly paramount in this[32]. In addition, patients with a genetic condition called hyper-IgE syndrome have been very recently demonstrated to show signs of coronary subclinical atherosclerosis[33]. Interestingly, our group has demonstrated that the mast cell stabilizer cromolyn acts in a protective manner in atherosclerosis experimental studies[34]. Therefore, a mast cell stabilization approach may be an interesting preventive strategy in individuals who show high circulating IgE levels, but who have not yet been diagnosed with cardiovascular disease syndromes.

As mentioned before, mast cell activation implies release of their granular material. When it comes to their protease secretome, chymase and tryptase are widely acclaimed as the most abundant as well as the most characteristic components of mast cell granules[35]. In our study, human atherosclerotic tissue mast cells show remarkable diversity in their chymase and tryptase content. Experimental mouse models and human observations have previously disclosed the adverse effects exerted by each individual protease in cardiovascular syndromes and suggest specific protease inhibition as a beneficial strategy[36]. One must keep in mind however, that chymase and tryptase can act differentially in the surrounding microenvironment, and that the balance between the two may differently affect plaque characteristics. It seems that while they appear to enhance inflammation at a full-blown or chronic degranulation scale, they can also, under circumstances, promote wound healing[37]. It is important to also note here that the number of patient samples in this analysis was limited as to draw any firm conclusions on the exact protease content and release. Nevertheless, we confirmed that most intraplaque mast cells in humans contain both tryptase and chymase, with an opening for additional studies on human intraplaque mast cells and their secretome. A possible analysis in the concentration of chymase and tryptase levels within the plaque or in the blood of these patients may shed more light into the degranulating material of

these cells.

In conclusion, in this brief human analysis we made use of flow cytometry or the first time, to characterize mast cells finside the atherosclerotic plaques of humans. We confirm that the major pathway for the activation of mast cells inside the plaque tissue is IgE-mediated and that intraplaque mast cells are highly activated and vary in their protease content. This is particularly important since it suggests that already available modes of therapy like mast cell stabilization agents[38], or anti-IgE treatment[39], may prove beneficial in patients suffering from atherosclerosis. In the future, it would be intriguing to assess the plaque stage of these human samples, to gain more information on the exact function of this interesting cell type. We hope that bigger scale patient studies and analysis of more characterization markers, for instance through mass spectrometry, will reveal new pathways *via* which mast cells may act in atherosclerosis, opening thus new ways to interfere in cardiovascular disease.

Reference list:

1. Benjamin, E. J. *et al.* Heart Disease and Stroke Statistics-2017 Update: A Report From the American Heart Association. *Circulation* **135,** e146–e603 (2017).
2. Bot, I., Shi, G.-P. & Kovanen, P. T. Mast cells as effectors in atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* **35,** 265–271 (2015).
3. Amin, K. The role of mast cells in allergic inflammation. *Respir. Med.* **106,** 9–14 (2012).
4. Shi, G.-P., Bot, I. & Kovanen, P. T. Mast cells in human and experimental cardiometabolic diseases. *Nat. Rev. Cardiol.* **12,** 643–658 (2015).
5. Kaartinen, M., Penttila, a. & Kovanen, P. T. Accumulation of activated mast cells in the shoulder region of human coronary atheroma, the predilection site of atheromatous rupture. *Circulation* **90,** 1669–1678 (1994).
6. Kovanen, P. T., Kaartinen, M. & Paavonen, T. Infiltrates of activated mast cells at the site of coronary atheromatous erosion or rupture in myocardial infarction. *Circulation* **92,** 1084–1088 (1995).
7. Bot, I. & Biessen, E. A. L. Mast cells in atherosclerosis. *Thromb. Haemost.* **106,** 820–826 (2011).
8. Ramalho, L. S. *et al.* Role of mast cell chymase and tryptase in the progression of atherosclerosis: study in 44 autopsied cases. *Ann. Diagn. Pathol.* **17,** 28–31 (2013).
9. Mayranpaa, M. I., Heikkila, H. M., Lindstedt, K. A., Walls, A. F. & Kovanen, P. T. Desquamation of human coronary artery endothelium by human mast cell proteases: implications for plaque erosion. *Coron. Artery Dis.* **17,** 611–621 (2006).
10. Kaartinen, M., Penttila, A. & Kovanen, P. T. Mast cells accompany microvessels in human coronary atheromas: implications for intimal neovascularization and hemorrhage. *Atherosclerosis* **123,** 123–131 (1996).
11. Kaartinen, M., Penttila, A. & Kovanen, P. T. Mast cells of two types differing in neutral protease composition in the human aortic intima. Demonstration of tryptase- and tryptase/chymase-containing mast cells in normal intimas, fatty streaks, and the shoulder region of atheromas. *Arterioscler. Thromb. a J. Vasc. Biol.* **14,** 966–972 (1994).
12. Wei, C.-C. *et al.* Mast cell chymase limits the cardiac efficacy of Ang I-converting enzyme inhibitor therapy in rodents. *J. Clin. Invest.* **120,** 1229–1239 (2010).
13. Somasundaram, P. *et al.* Mast cell tryptase may modulate endothelial cell phenotype in healing myocardial infarcts. *J. Pathol.* **205,** 102–111 (2005).
14. Rohm, I. *et al.* Increased Number of Mast Cells in Atherosclerotic Lesions Correlates with the Presence of Myeloid but not Plasmacytoid Dendritic Cells as well as Pro-inflammatory T Cells. *Clin. Lab.* **62,** 2293–2303 (2016).
15. Willems, S. *et al.* Mast cells in human carotid atherosclerotic plaques are associated with intraplaque microvessel density and the occurrence of future cardiovascular events. *Eur. Heart J.* **34,** 3699–3706 (2013).
16. Wang, J. *et al.* IgE stimulates human and mouse arterial cell apoptosis and cytokine expression and promotes atherogenesis in Apoe-/- mice. *J. Clin. Invest.* **121,** 3564–3577 (2011).
17. Guo, X. *et al.* Serum IgE levels are associated with coronary artery disease severity. *Atherosclerosis* **251,** 355–360 (2016).
18. Kelley, J. *et al.* Mast cell activation by lipoproteins. *Methods Mol. Biol.* **315,** 341–348 (2006).
19. Yu, Y., Blokhuis, B. R., Garssen, J. & Redegeld, F. A. Non-IgE mediated mast cell activation. *Eur. J. Pharmacol.* **778,** 33–43 (2016).
20. Meng, Z. *et al.* Oxidized low-density lipoprotein induces inflammatory responses in

cultured human mast cells via Toll-like receptor 4. *Cell. Physiol. Biochem.* **31,** 842–53 (2013).

21. de Vries, M. R. *et al.* Complement factor C5a as mast cell activator mediates vascular remodelling in vein graft disease. *Cardiovasc. Res.* **97,** 311–320 (2013).
22. Bot, I. *et al.* The neuropeptide substance P mediates adventitial mast cell activation and induces intraplaque hemorrhage in advanced atherosclerosis. *Circ. Res.* **106,** 89–92 (2010).
23. Van Brussel, I. *et al.* Fluorescent activated cell sorting: an effective approach to study dendritic cell subsets in human atherosclerotic plaques. *J. Immunol. Methods* **417,** 76–85 (2015).
24. Burd, P. R. *et al.* Interleukin 3-dependent and -independent mast cells stimulated with IgE and antigen express multiple cytokines. *J. Exp. Med.* **170,** 245–257 (1989).
25. Tsai, M. *et al.* Induction of mast cell proliferation, maturation, and heparin synthesis by the rat c-kit ligand, stem cell factor. *Proc. Natl. Acad. Sci. U. S. A.* **88,** 6382–6386 (1991).
26. Kraft, S. *et al.* The tetraspanin CD63 is required for efficient IgE-mediated mast cell degranulation and anaphylaxis. *J. Immunol.* **191,** 2871–2878 (2013).
27. Sinkiewicz, W. *et al.* Immunoglobulin E as a marker of the atherothrombotic process in patients with acute myocardial infarction. *Cardiol. J.* **14,** 266–273 (2007).
28. Langer, R. D., Criqui, M. H., Feigelson, H. S., McCann, T. J. & Hamburger, R. N. IgE predicts future nonfatal myocardial infarction in men. *J. Clin. Epidemiol.* **49,** 203–209 (1996).
29. Lippi, G., Cervellin, G. & Sanchis-Gomar, F. Immunoglobulin E (IgE) and ischemic heart disease. Which came first, the chicken or the egg? *Ann. Med.* **46,** 456–463 (2014).
30. Insull, W. J. The pathology of atherosclerosis: plaque development and plaque responses to medical treatment. *Am. J. Med.* **122,** S3–S14 (2009).
31. Laske, N., Bunikowski, R. & Niggemann, B. Extraordinarily high serum IgE levels and consequences for atopic phenotypes. *Ann. Allergy. Asthma Immunol.* **91,** 202–204 (2003).
32. Wang, L. *et al.* Allergic asthma accelerates atherosclerosis dependent on Th2 and Th17 in apolipoprotein E deficient mice. *J. Mol. Cell. Cardiol.* **72,** 20–27 (2014).
33. Abd-Elmoniem, K. Z. *et al.* Coronary atherosclerosis and dilation in hyper IgE syndrome patients: Depiction by magnetic resonance vessel wall imaging and pathological correlation. *Atherosclerosis* **258,** 20–25 (2017).
34. Bot, I. *et al.* Perivascular mast cells promote atherogenesis and induce plaque destabilization in apolipoprotein E-deficient mice. *Circulation* **115,** 2516–2525 (2007).
35. Caughey, G. H. Mast cell proteases as pharmacological targets. *Eur. J. Pharmacol.* **778,** 44–55 (2016).
36. He, A. & Shi, G.-P. Mast cell chymase and tryptase as targets for cardiovascular and metabolic diseases. *Curr. Pharm. Des.* **19,** 1114–1125 (2013).
37. Douaiher, J. *et al.* Development of mast cells and importance of their tryptase and chymase serine proteases in inflammation and wound healing. *Adv. Immunol.* **122,** 211–252 (2014).
38. Finn, D. F. & Walsh, J. J. Twenty-first century mast cell stabilizers. *Br. J. Pharmacol.* **170,** 23–37 (2013).
39. Logsdon, S. L. & Oettgen, H. C. Anti-IgE therapy: clinical utility and mechanistic insights. *Curr. Top. Microbiol. Immunol.* **388,** 39–61 (2015).

Chapter 7

Mast cell depletion in advanced atherosclerosis does not induce plaque regression

Manuscript in preparation

[1]Eva Kritikou
[1]Thomas van der Heijden
[1]Gijs H.M. van Puijvelde
[1]Maarten Swart
[1]Mara J. Kröner
[1]Johan Kuiper
[1]Ilze Bot

[1] Division of Biopharmaceutics, Leiden Academic Centre for Drug Research, Leiden University, Leiden, The Netherlands

Abstract

Regression of atherosclerotic plaques is a continuous challenge in the prevention of acute cardiovascular syndromes. While many pharmacological approaches have proven successful in arresting progressing atherosclerosis, it is still complicated to reverse the process of plaque formation and reduce pre-formed stenotic tissue in arteries. Mast cells are plaque components, known for their pro-inflammatory role during atherosclerosis progression. Numerous studies have shown that this cell type accumulates inside atherosclerotic plaques and their activation, and subsequent degranulation, can enhance plaque development, while their stabilization hampers plaque progression. However, mast cells have also been implicated in atherosclerosis regression and in this project, we aimed to determine whether mast cell depletion can induce plaque regression. We made use of the DTR-conditional depletion model, to abolish the presence of mast cells in RMB-LDLr$^{-/-}$ mice while simultaneously altering the food content from high-fat to lipid-low diet. We did not observe any differences in atherosclerotic plaque size, as well as macrophage or smooth muscle cell content, indicating that mast cell depletion in this setup did not induce plaque regression. The absence of mast cells however, did markedly reduce the amount of infiltrating neutrophils and collagen content in the plaque area. In conclusion, depletion of mast cells is not an optimal therapeutic target for the reversion of pre-established atherosclerosis.

1. Introduction

Acute cardiovascular events, generally caused by atherosclerotic plaque progression in large arteries, are identified as the major cause of death in Western societies[1]. Atherosclerosis progresses at a chronic, yet silent, fashion that can remain undetected for years; until the plaque reaches an advanced stage, where it may either destabilize, or rupture, leading to a myocardial infarction or a stroke[2]. Up to date, the only therapeutic method available to eradicate an atherosclerotic plaque, is surgical intervention, for instance through coronary angioplasty with or without stent-placement, which can however lead to secondary (re)stenosis[3]. A possible reduction of the atherosclerotic plaque size, upon induction of plaque regression *via* non-invasive therapy, would help in the struggle against unwanted side-effects. From a pharmacological perspective, statin use can decrease the (very) low density-lipoprotein content, providing plaque stability. Still, lipoprotein fraction modulation alone, without taking the inflammatory context into account, is not able to induce plaque regression[4,5]. Previously, it has been shown in mice, that lowering of the lipid diet content in combination with anti-inflammatory treatment, can induce regression of atherosclerotic plaques[6,7]. However, until now no such method has been successfully applied on human subjects[8]. In a recent experimental model study, successful plaque regression was achieved in LDL-receptor$^{-/-}$ mice by targeting the adaptive immune response, through the inhibition of the co-stimulatory pathway triggered by OX40 and OX40-ligand[7]. Interestingly, this intervention affected also the mast cell content of the atherosclerotic plaque, which appeared reduced in number and activation status. This event was of particular importance, considering the role that mast cells play in atherosclerosis.

Mast cells accumulate within the plaque tissue in the intima and adventitia[9] and promote disease progression and plaque destabilization[10]. The adverse effect of mast cells is mainly attributed to their degranulating nature, which leads to the release of pro-inflammatory mediators[11] such as cytokines and proteases. Our group has previously found that apoE$^{-/-}$ mice develop increased plaques upon systemic mast cell activation, an effect which was reversed by using a mast cell stabilizer[12]. Furthermore, mast cell deficient LDLr$^{-/-}$/Kit$^{W\text{-}sh/W\text{-}sh}$ mice show lower plaque size and lipid deposition, as compared to control LDLr$^{-/-}$ mice[13]. Importantly, in a clinical study where human plaque specimens from carotid arteries were analysed, mast cells were found to be the only inflammatory cell type that positively correlated with increased intra-plaque haemorrhage and future cardiovascular events[14]. For these reasons, mast cell targeting appears to be significantly interesting as a possible method to control atherosclerosis.

Therefore, in this study we aimed to assess the therapeutic potential that mast

cell deficiency may exert, in combination with a lipid lowering diet.

2. Materials & Methods

2.1 Animals

All animal work was performed according to the guidelines of the European Parliament Directive 2010/63EU and all experimental work was approved by the Animal Ethics committee of Leiden University. The mice were bred and housed in the facility under *ad libitum* water and food supplies. The red mast cell and basophil mouse strain (RMB mice, official name: B6.Ms4a2^{tm1Mal}) was provided by the laboratory of dr. P. Launay INSERM U1149, Paris, France[15]. These mice express the simian diphtheria toxin receptor (DTR)[16] under control of the FcεR1β gene promoter, a receptor which is specifically expressed by mast cells[17]. Injection of diphtheria toxin (DT) will therefore induce apoptosis of all FcεR1β expressing cells. RMB mice were subsequently crossed with LDLr$^{-/-}$ mice (Jackson Laboratories, USA) to obtain atherosclerosis-prone RMB-LDLr$^{-/-}$ mice. LDLr$^{-/-}$ mice were included in the study as a control group (control-LDLr$^{-/-}$) for DT-mediated side effects[18].

2.2 Atherosclerosis

Male RMB-LDLr$^{-/-}$ (n=12/group), and control-LDLr$^{-/-}$ (n=6) of average age 19-20 weeks were fed a Western type diet (WTD) [0.25% cholesterol, 15% cocoa butter; Special Diet Services, Essex, UK] for 10 weeks, to develop atherosclerosis. After the 10-week WTD period, a baseline group of mice was sacrificed for assessment of the high-lipid diet effects. The remaining groups of mice were placed on a low-lipid (chow) diet for a subsequent period of 6 weeks. Simultaneously the DT/PBS injection scheme was initiated. Diphtheria toxin was injected intraperitoneally (0.5mg/mL) in order to achieve specific depletion of all FcεR1β-expressing cells. DT was injected 3 times within the first week of the depletion period, for optimal cell clearance (boost period). After the first week, DT was injected at a maintenance fashion every 13 days. Before the baseline sacrifice and DT-administration period, all mice were randomized according to their age, weight and total serum cholesterol levels. All remaining mice were monitored for body weight changes throughout the compound administration period and until the endpoint of the experiment. After the 6-week chow period all mice were anesthetized by subcutaneous injections using ketamine (100mg/mL), sedazine (25mg/mL) and atropine (0.5mg/mL) and their vascular system was perfused by PBS administration at a continuous low flow *via* heart puncture in the left ventricle.

2.3 Total cholesterol analysis

Serum was obtained upon separation of blood, collected through the tail-vein. At the experimental endpoint, serum was collected from blood collected *via* the eye vein. Serum separation was achieved by centrifugation at 8.000 rpm for 10 minutes. Total cholesterol levels in the serum were assessed using an enzymatic colorimetric assay with Precipath as an internal control (Roche Diagnostics).

2.4 Immunohistochemistry

The hearts of all mice were fixed in formalin for 24 hours and subsequently dissected below the atria. All hearts were embedded in O.C.T. compound (Sakura) and sectioned horizontally to the aortic axis and towards the aortic arch. Upon identification of the aortic root, defined by the

trivalve leaflets, 10μm sections were collected. Mean plaque size (in μm^2) was calculated for three sequential sections, displaying the highest plaque content, using an Oil-red-O staining (Sigma Aldrich), which is specific for neutral lipid marking. Mast cell and neutrophil content was manually quantified following a Naphthol AS-D chloro-acetate esterase kit (Sigma Aldrich). The distinction of neutrophils from mast cells was performed according to their cellular characteristics, specifically the pink granular cytoplasm and lobular nucleus. Plaque macrophages were stained with a MOMA-2 antibody at a 1:1000 concentration (rat IgG2b, Serotec Ltd.). Smooth muscle cell quantification was performed following a staining with α-smooth muscle actin (αSMA) at a concentration of 1:1000 (Abcam). Intraplaque collagen was assessed using a Masson's trichrome staining kit (Sigma Aldrich). T cell content in the aortic root was determined after a CD3 staining at a 1:150 concentration (clone SP7, ThermoScientific). All analyses were performed on a Leica DM-RE microscope and staining quantification levels were measured using a Leica QWin software (Leica, Imaging Systems, UK) and through blinded independent analysis.

2.5 Flow cytometry

Blood, spleen and heart lymph node tissues were harvested for further analysis of their immune cell populations. To obtain blood leukocytes, red blood cell lysis was performed with a solution of 0.1mM EDTA, 10mM $NaHCO_3$, 1mM NH_4Cl, pH=7.2. Splenocytes as well as cells from the draining heart lymph nodes were obtained upon organ grinding through a 70μm filter and subsequent erythrocyte lysis. All cells were stained with fluorescently labeled antibodies against the markers of interest (**Table 1**). All antibodies were used at a concentration of 0.1μg/sample. The flow cytometry experiments were performed with a FACS Canto II and data were analyzed using a FlowJo software.

Antibody	Fluorochrome	Clone	Company
CD11b	eFluor 450	M1/70	Ebioscience
Ly6G	PE	1A8	BD Biosciences
NK1.1	FITC	PK136	Ebioscience
FcεR1α	PercP Cy5.5	MAR-1	Biolegend
CD117	APC	2B8	Ebioscience
CD4	eFluor 450	GK1.5	Ebioscience
CD8a	PercP	53-6.7	BD Biosciences

Table 1: Flow cytometry antibodies

2.6 Statistics

Data collected were analyzed for normal distribution and are presented as mean±SEM. For the analysis of one variable between more than two groups a one-way ANOVA was performed, with a Bonferroni post-test for multiple comparisons. For the analysis of one variable between two individual groups, a 2-tailed Student's *t*-test was used, with the Bonferroni post-test for multiple comparisons. The probability for all tests was set to 0.05 with lower values considered significant ($P<0.05$).

3. Results

3.1 Conditional depletion of mast cells using the Diphtheria toxin mouse model

In this study, we aimed to evaluate effects of mast cell depletion on atherosclerosis, in combination with a lipid-lowering diet. After a 10-week period of WTD, a baseline group of RMB-LDLr$^{-/-}$ mice was sacrificed, to determine baseline plaque characteristics. The remaining groups of mice were switched to a chow diet while injected with DT or PBS for a period of 6 weeks. An additional group of LDLr$^{-/-}$ mice served as controls to estimate possible toxic effects of diphtheria toxin. Through the injection period we monitored all three groups of mice (PBS, DT & control) for possible changes in body weight. However, we did not observe any substantial differences in body weight development between the control group and the PBS or DT groups (**Figure 1A**). This indicated that administration of DT in such fashion does not elicit any toxic side effects and hence we proceeded with comparing the baseline, PBS and DT groups. To ensure that the diet-reversal indeed reduced the circulating lipid content, we measured the total cholesterol levels in the serum at the end of the study. While the baseline group showed high total serum cholesterol, the PBS and DT groups displayed a significant reduction in their circulating cholesterol levels, as expected upon diet switch (**Figure 1B**, P<0.0001). We next aimed to assess the efficiency of DT to deplete mast cells, by specifically examining the presence of mast cells in the perivascular tissue of the aortic root in the mouse hearts; an area prone to develop atherosclerosis. Upon manual quantification of cell numbers, we observed that mast cells were absent in the DT group, as compared to the baseline and PBS groups of mice (**Figure 1C**, DT: 0.77±0.29 cells per section vs PBS: 12.83±1.94 cells per section, P<0.0001; vs baseline: 9.52±1.65 cells per section, P=0.0005). Representative pictures of the Naphthol AS D-chloroacetate esterase staining performed in the aortic root of the hearts demonstrated that in the DT group all mast cells were virtually absent (**Figure 1D**).

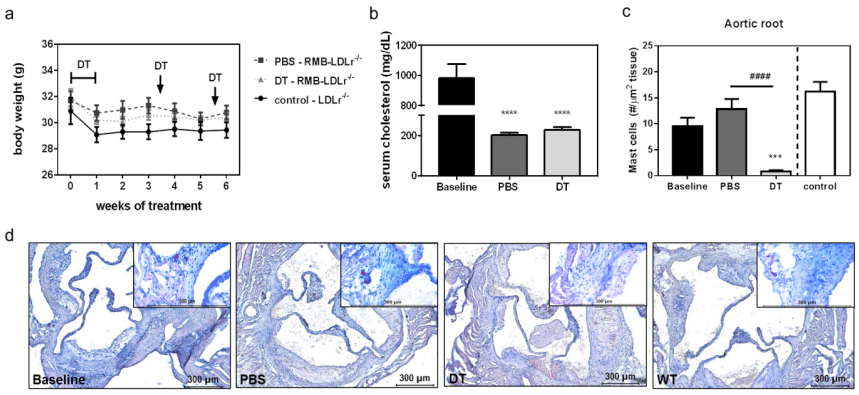


Figure 1: RMB-LDLr$^{-/-}$ conditional depletion using diphtheria toxin resulted in the abolishment of mast cells in the aortic root of the hearts. (A) Diphtheria toxin (DT) i/p injections (0.5mg/mL) did not evoke unspecific effects on the body weight of the DT or control group of mice, as compared to the PBS group. **(B)** Reversal from WTD to chow diet significantly reduced total cholesterol levels in the serum in both groups of mice (PBS & DT) as compared to the baseline group. **(C)** The number of mast cells in the aortic root of the heart after 6 weeks of DT-specific mast cell depletion was diminished, in comparison to the PBS group. **(D)** The number of mast cells was quantified with the use of a Naphthol AS-D chloro-acetate esterase staining; representative pictures of 10μm sections in the aortic root of the heart are shown. All values (n=12/grp) are depicted as mean±SEM. ****P<0.001, ****P<0.0001* compared to the baseline group; *####P<0.0001* compared to the PBS-treated group.

3.2 Mast cell depletion does not alter plaque size upon chow diet

Upon establishing the successful depletion of mast cells inside the atherosclerotic tissue, we moved on to characterize the impact of their absence in atherosclerotic plaque size. We used an ORO staining to visualize the lipid content of the plaque (**Figure 2A**) in the aortic root. However, quantification of the plaque size did not display any significant differences among the three groups of mice (**Figure 2B**, DT: $26*10^4 \pm 3*10^4$ μm^2, PBS: $30*10^4 \pm 2*10^4$ μm^2 baseline: $29*10^4 \pm 5*10^4$ μm^2, *P>0.05*). Subsequently, we assessed the macrophage content of the plaque with a MOMA-2 staining (**Figure 2C**). Diet reversal from WTD to chow revealed a sharp reduction in the intraplaque macrophage content of both the PBS and DT groups **(Figure 2D**, P<0.0001). Additionally, analysis of the plaque T cell content in the hearts was performed by an immunohistochemical staining against membrane protein CD3 (**Figure 2E**). We observed a significant decrease in T cell numbers in the plaques of the DT group as compared to the baseline; yet no difference was observed among DT and PBS mice (**Figure 2F**, DT: 12.2±1.4 cells vs baseline: 39.9±10.3 cells, P=0.012).

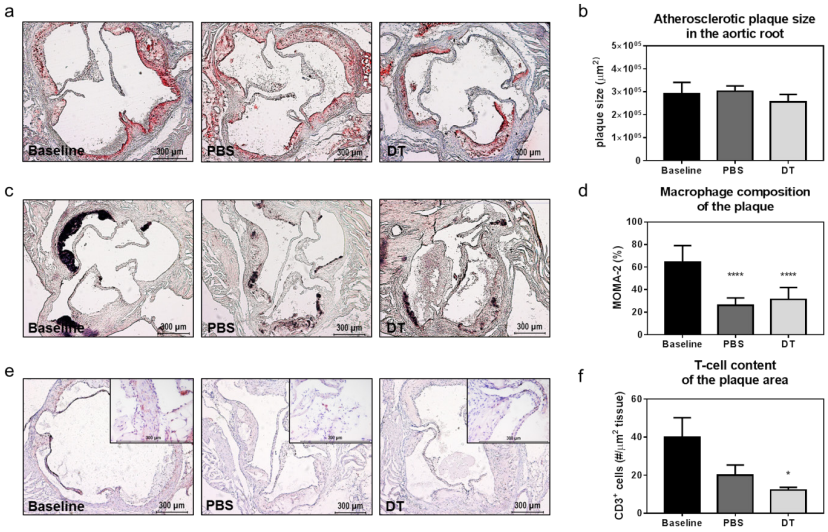


Figure 2: Conditional depletion of mast cells upon chow diet did not affect atherosclerotic plaque size, macrophage content or T cell number in the aortic root of the heart. (A) An Oil-Red-O staining in the aortic root was performed to determine the atherosclerotic plaque size among the groups; representative pictures are shown. **(B)** No significant difference was observed between the Baseline, PBS and DT groups. **(C)** The macrophage content of the atherosclerotic plaques was quantified using a MOMA-2 staining; example pictures of the staining are depicted for each group. **(D)** Diet reversal from WTD to chow, resulted in a substantial reduction in the macrophage plaque content, with no difference observed between the PBS and DT groups. **(E)** The number of T cells was assessed using a staining against CD3; representative pictures are shown. **(F)** The number of T cells present in the aortic root of DT mice was significantly reduced as compared to the baseline group, however no significant changes were observed between the DT and PBS groups. All values (n=12/grp) are depicted as mean±SEM. **P<0.05, ****P<0.0001* compared to the baseline group.

3.3 Atherosclerotic plaques appear less stable upon mast cell depletion

The collagen deposition, as measured by a Masson's Trichrome staining, in the plaques of the DT mice was found significantly reduced in comparison to the PBS group of mice (**Figure 3A**,**3B**, DT: $88*10^4 \pm 13*10^4$ µm^2 vs PBS: $136*10^4 \pm 17*10^4$ µm^2, P=0.044). No significant changes were detected when comparing the baseline and DT mice. Of note, no difference was observed in the necrotic core area of the plaques between the different groups of mice (DT:$53*10^4 \pm 11*10^4$ µm^2, PBS: $68*10^4 \pm 9*10^4$ µm^2, baseline: $58*10^4 \pm 15*10^4$ µm^2). We also determined the smooth muscle cell (SMC) content of all groups (**Figure 3C**), however, this did not differ between the three groups of mice (**Figure 3D**).

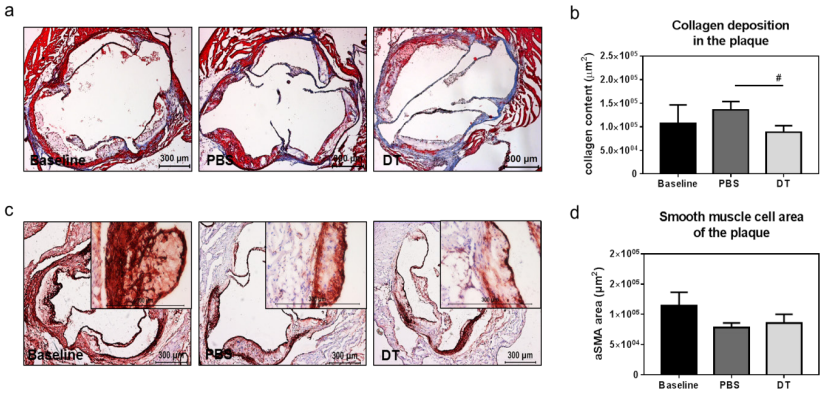


Figure 3: Collagen deposition in the aortic root of the hearts was reduced upon mast cell depletion. (A) The collagen content of the aortic root was quantified using a Masson's Trichrome staining; representative pictures are presented. **(B)** The deposition of collagen inside the atherosclerotic plaques of DT mice was found significantly reduced as compared to the PBS group of mice. **(C)** Quantification of the smooth muscle cell content in the plaque area of the heart was performed using an αSMA staining; representative pictures per group are shown. **(D)** No difference was observed between the PBS and DT groups in the total smooth muscle cell area of the aortic root. All values (n=12/grp) are depicted as mean±SEM. *#P<0.05* compared to the PBS treated group.

3.4 The neutrophil content is negatively affected by the absence of mast cells in the plaque

As mast cells were previously established to induce neutrophil chemotaxis[19], we analysed the neutrophil content in the circulation and the atherosclerotic plaque of all mice. We did not observe any differences in the percentage of circulating neutrophils, defined as $Ly6C^+/CD11b^+Ly6G^-/NK1.1^-$ cells (**Figure 4A**). However, in line with previous data from our group[19], we observed a significant reduction in the intraplaque neutrophil numbers upon mast cell depletion, as compared to both the PBS and baseline groups (**Figure 4B**, DT: 5.4±1.1 cells vs PBS: 17.7±2.3 cells, P<0.0001; vs baseline: 16.3±1.9 cells, P=0.0005).

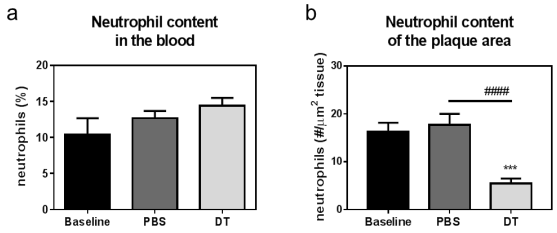


Figure 4: The neutrophil content of the aortic root was significantly diminished following the depletion of mast cells upon chow diet. (A) The proportion of neutrophils present in the circulation was not altered among the PBS and DT groups. **(B)** The number of neutrophils accumulating in the aortic root of DT treated mice was highly reduced during mast cell depletion, as compared to the PBS and baseline groups. All values (n=12/grp) are depicted as mean±SEM. ****P<0.001* compared to the baseline group; *####P<0.0001* compared to the PBS-treated group.

3.5 Mast cell absence reduces the levels of $CD4^+$ and $CD8^+$ T cells in the heart lymph nodes

Since the inflammatory cell content of the atherosclerotic plaque is in direct communication with the proximal heart lymph nodes (hLN)[20], an area rich in T cells, we aimed to analyze the local $CD4^+$ and $CD8^+$ T cell content using flow cytometry. The $CD4^+$ percentage in the draining hLNs was significantly reduced in the DT as compared to the PBS group (**Figure 5A**, DT: 17.75±0.59 % as compared to PBS: 20.69±0.63 %, P=0.003). However, no difference was observed among the percentages of $CD4^+$ T cells circulating in the blood (**Figure 5B**). Along the same line, $CD8^+$ T cells appeared at a substantially lower percentage in the hLNs of the DT mice as compared to the PBS mice (**Figure 5C**, DT: 15.6±0.9 % compared to PBS: 18.9±0.9 %, P=0.020). Finally, no differences were observed among the three groups in the levels of circulating $CD8^+$ T cells (**Figure 5D**).

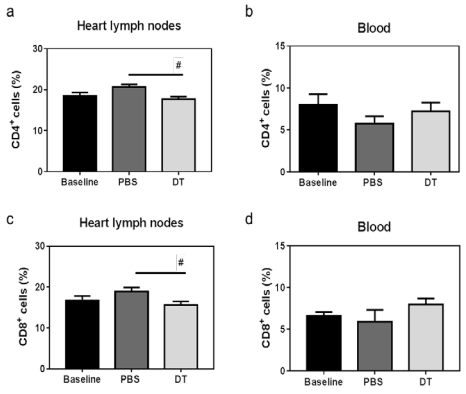


Figure 5: CD4⁺ and CD8⁺ T cell levels were reduced in the heart lymph nodes of mast cell depleted mice. (A) The percentage of CD4⁺ T cells in the heart lymph nodes was significantly reduced in the DT group as compared to the PBS. **(B)** The amount of circulating CD4⁺ T cells did not differ between the groups of mice. **(C)** The CD8⁺ T cell percentage in the heart lymph nodes was decreased in the DT group as compared to the PBS. **(D)** Blood CD8⁺ T cells were not altered upon DT treatment, in comparison to the other two groups of mice. All values (n=12/grp) are depicted as mean±SEM. *#P<0.05* compared to the PBS-treated group.

4. Discussion

In the present study, we aimed to assess the possible therapeutic effect of mast cell depletion on pre-existing atherosclerotic plaques, in the presence of a lipid lowering diet. We confirmed that the switch from WTD to chow diet reduced the total cholesterol levels in the circulation and furthermore, that DT treatment depleted the mast cell content from the tissues, including the aortic root of the heart. Despite the successful depletion of mast cells, we did not detect regression in the atherosclerotic plaque size of the aortic root; indicating that mast cell depletion in this context is not relevant as therapeutic means for the regression of atherosclerosis.

Macrophages were markedly affected by the diet reversal in both chow-fed groups of mice, similarly as described previously[7]; but the absence of mast cells did not have any additional effect on the plaque macrophage content.

In contrast, collagen deposition in the plaques was found reduced in the mast cell depleted mice. At first glance this seems surprising, considering that mast cell chymase inhibition has been reported to increase the intraplaque collagen content in apoE$^{-/-}$ mice[21]. In addition, mast cell degranulation products have been implicated in the degradation of extracellular matrix components[11], with mast cell deficient LDLr$^{-/-}$/Kit$^{W\text{-}sh/W\text{-}sh}$ mice showing higher collagen levels as compared to their wild-type counterparts[13]. In line with the above, mast cell stabilization with tranilast was reported to increase collagen deposition in a diabetes study[22]. However, mast cell tryptase has been noted to increase collagen synthesis in renal fibrosis[23]. This suggests that the level and quality of degranulating mast cell products can have a substantially different effect on the collagen deposition process. Furthermore, mast cells have been proven to have a regenerative capacity under tissue repair conditions. For instance, mast cell infiltration and degranulation mediators in the myocardium has been proven crucial in extracellular

matrix remodelling[24], during the healing phase following an infarct episode. In contrast to the observed collagen reduction, the smooth muscle cell content of the plaques was not altered upon mast cell depletion. While this result may seem inexplicable, smooth muscle cell α-actin is a cytoskeletal protein expressed in terminally differentiated arterial smooth muscle cells, and not in the SMCs undergoing proliferation[25]. However, it is the proliferative state of SMCs that is characterized by collagen production inside the atherosclerotic plaques[26]. The collagen reduction observed here may, thus, be linked primarily to proliferative smooth muscle cells and, therefore, not directly determined by α-SMA.

The neutrophil intraplaque content was also found highly reduced upon mast cell depletion. Mast cell tryptase has been previously reported to attract neutrophils in the surrounding tissue, through endothelial mediated cytokine release[27]. Furthermore, recently our group has observed that neutrophil infiltration within atherosclerotic plaques is greatly affected by mast cells and particularly through their release of chemokine CXCL1 that can direct neutrophils inside the plaques by acting on their CXCR2 receptor[19]. Moreover, a close relationship between mast cells and neutrophils was also reported in rheumatoid arthritis[28].

In addition, the total T cell population appeared significantly reduced in the hearts of chow-fed mice that did not contain mast cells, as compared to WTD-fed mice with mast cells. Lately it is being proposed that mast cells can interact with T cells in a direct fashion[29]. In the case of atherosclerosis, stabilization of mast cells in LDLr$^{-/-}$ mice has been reported to reduce CD4^{+} T cell numbers in the plaques[30], whereas the intraplaque amount of mast cells was positively associated with the number of CD8^{+} T cells in human atherosclerotic specimens[31]. Therefore, it could be that the physical presence of mast cells is needed, to a certain extend, for T cells to infiltrate or survive within the atherosclerotic plaques. Interestingly, in the draining heart lymph nodes, both CD4^{+} and CD8^{+} T cell populations appeared markedly reduced in the absence of mast cells, suggesting that mast cells may shape the immune response of secondary lymphoid organs. However, no effect was observed in the circulating rate of both CD4^{+} and CD8^{+} T cells. The fact that the absence of mast cells seems to affect mainly the lymph node population that is adjacent to the atherosclerotic site, and less the systemic T cell response, indicates that the observed effects are local and atherosclerosis specific.

Overall, the atherosclerotic plaques of mice upon lipid lowering appeared to contain similar amounts of macrophages, T cells and smooth muscle cells, regardless of whether mast cells were present or not. However, the absence of mast cells significantly affected the amount of collagen deposited in the atherosclerotic area as well as the local neutrophil infiltration. In this experimental setup, mast cell depletion did not induce atherosclerosis regression upon a lipid-lowering diet. It is worth mentioning

that intervention on the $CD3^+$ axis has been previously shown to induce atherosclerosis regression[6]. Be that as it may, in regressing plaques it is equally crucial for foam cells to emigrate in the draining lymph nodes[32], where they can act on the local T cell response. In this study, we saw that while mast cells were able to shape the $CD4^+/CD8^+$ lymph node content they did not regulate macrophage emigration from the plaques, and therefore plaque regression. Ultimately, even though mast cells seem to firmly assist atherosclerosis regression[7] they do not seem able to trigger it. Nevertheless, the above data indicate that although mast cells are regarded as classical pro-inflammatory effectors, they may also have protective effects that favour plaque stabilization. Therefore, in atherosclerosis it may be optimal to refine individual pathways of mast cell action and selectively intervene on the adverse ones in order to successfully interfere in disease progression.

Reference list:

1. Benjamin, E. J. *et al.* Heart Disease and Stroke Statistics-2017 Update: A Report From the American Heart Association. *Circulation* **135,** e146–e603 (2017).
2. Hansson, G. K., Libby, P. & Tabas, I. Inflammation and plaque vulnerability. *J. Intern. Med.* **278,** 483–493 (2015).
3. Kim, M. S. & Dean, L. S. In-stent restenosis. *Cardiovasc. Ther.* **29,** 190–198 (2011).
4. Nissen, S. E. *et al.* Effect of very high-intensity statin therapy on regression of coronary atherosclerosis: the ASTEROID trial. *JAMA* **295,** 1556–1565 (2006).
5. Hewing, B. & Fisher, E. A. Preclinical mouse models and methods for the discovery of the causes and treatments of atherosclerosis. *Expert Opin. Drug Discov.* **7,** 207–216 (2012).
6. Kita, T. *et al.* Regression of atherosclerosis with anti-CD3 antibody via augmenting a regulatory T-cell response in mice. *Cardiovasc. Res.* **102,** 107–117 (2014).
7. Foks, A. C. *et al.* Interruption of the OX40-OX40 ligand pathway in LDL receptor-deficient mice causes regression of atherosclerosis. *J. Immunol.* **191,** 4573–4580 (2013).
8. Feig, J. E. Regression of atherosclerosis: insights from animal and clinical studies. *Ann. Glob. Heal.* **80,** 13–23 (2014).
9. Laine, P. *et al.* Association Between Myocardial Infarction and the Mast Cells in the Adventitia of the Infarct-Related Coronary Artery. *Circulation* **99,** 361–369 (1999).
10. Bot, I., Shi, G.-P. & Kovanen, P. T. Mast cells as effectors in atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* **35,** 265–271 (2015).
11. Kovanen, P. T., Kaartinen, M. & Paavonen, T. Infiltrates of activated mast cells at the site of coronary atheromatous erosion or rupture in myocardial infarction. *Circulation* **92,** 1084–1088 (1995).
12. Bot, I. *et al.* Perivascular mast cells promote atherogenesis and induce plaque destabilization in apolipoprotein E-deficient mice. *Circulation* **115,** 2516–2525 (2007).
13. Sun, J. *et al.* Mast cells promote atherosclerosis by releasing proinflammatory cytokines. *Nat. Med.* **13,** 719–724 (2007).
14. Willems, S. *et al.* Mast cells in human carotid atherosclerotic plaques are associated with intraplaque microvessel density and the occurrence of future cardiovascular events. *Eur. Heart J.* **34,** 3699–3706 (2013).
15. Dahdah, A. *et al.* Mast cells aggravate sepsis by inhibiting peritoneal macrophage phagocytosis. *J. Clin. Invest.* **124,** 4577–4589 (2014).
16. Saito, M. *et al.* Diphtheria toxin receptor-mediated conditional and targeted cell ablation in transgenic mice. *Nat. Biotechnol.* **19,** 746–750 (2001).
17. Beaven, M. A. & Baumgartner, R. A. Downstream signals initiated in mast cells by Fc epsilon RI and other receptors. *Curr. Opin. Immunol.* **8,** 766–772 (1996).
18. Christiaansen, A. F., Boggiatto, P. M. & Varga, S. M. Limitations of Foxp3(+) Treg depletion following viral infection in DEREG mice. *J. Immunol. Methods* **406,** 58–65 (2014).
19. Wezel, A. *et al.* Mast cells mediate neutrophil recruitment during atherosclerotic plaque progression. *Atherosclerosis* **241,** 289–296 (2015).
20. Randolph, G. J. Emigration of monocyte-derived cells to lymph nodes during resolution of inflammation and its failure in atherosclerosis. *Curr. Opin. Lipidol.* **19,** 462–468 (2008).
21. Bot, I. *et al.* Mast cell chymase inhibition reduces atherosclerotic plaque progression and improves plaque stability in ApoE-/- mice. *Cardiovasc. Res.* **89,** 244–252 (2011).
22. Jones, S. E., Gilbert, R. E. & Kelly, D. J. Tranilast reduces mesenteric vascular collagen deposition and chymase-positive mast cells in experimental diabetes. *J. Diabetes Complications* **18,** 309–315 (2004).
23. Kondo, S. *et al.* Role of mast cell tryptase in renal interstitial fibrosis. *J. Am. Soc. Nephrol.*

12, 1668–1676 (2001).

24. Somasundaram, P. *et al.* Mast cell tryptase may modulate endothelial cell phenotype in healing myocardial infarcts. *J. Pathol.* **205,** 102–111 (2005).
25. Thyberg, J. Differentiated properties and proliferation of arterial smooth muscle cells in culture. *Int. Rev. Cytol.* **169,** 183–265 (1996).
26. Adiguzel, E., Ahmad, P. J., Franco, C. & Bendeck, M. P. Collagens in the progression and complications of atherosclerosis. *Vasc. Med.* **14,** 73–89 (2009).
27. Compton, S. J., Cairns, J. A., Holgate, S. T. & Walls, A. F. Human mast cell tryptase stimulates the release of an IL-8-dependent neutrophil chemotactic activity from human umbilical vein endothelial cells (HUVEC). *Clin. Exp. Immunol.* **121,** 31–36 (2000).
28. Pimentel, T. A., Sampaio, A. L. F., D'Acquisto, F., Perretti, M. & Oliani, S. M. An essential role for mast cells as modulators of neutrophils influx in collagen-induced arthritis in the mouse. *Lab. Invest.* **91,** 33–42 (2011).
29. Bulfone-Paus, S. & Bahri, R. Mast Cells as Regulators of T Cell Responses. *Front. Immunol.* **6,** 394 (2015).
30. Wang, J. *et al.* Pharmaceutical stabilization of mast cells attenuates experimental atherogenesis in low-density lipoprotein receptor-deficient mice. *Atherosclerosis* **229,** 304–309 (2013).
31. Rohm, I. *et al.* Increased Number of Mast Cells in Atherosclerotic Lesions Correlates with the Presence of Myeloid but not Plasmacytoid Dendritic Cells as well as Pro-inflammatory T Cells. *Clin. Lab.* **62,** 2293–2303 (2016).
32. Trogan, E. *et al.* Gene expression changes in foam cells and the role of chemokine receptor CCR7 during atherosclerosis regression in ApoE-deficient mice. *Proc. Natl. Acad. Sci. U. S. A.* **103,** 3781–3786 (2006).

7

Chapter 8

General discussion - Future perspectives

Synopsis

Atherosclerosis is the most prevailing underlying pathology responsible for the increased mortality rates due to acute cardiovascular syndromes[1]. Cardiovascular disease (CVD) was accounted for 1 in 3 deaths in US adults over the year 2014, with approximately 1 death incidence occurring every 40 seconds[2]. In Europe, CVD is the cause of 45% of all deaths, according to the 2017 statistics rates[3]. Despite the misconception that cancer is the leading cause of death nowadays, mortality due to CVD is actually higher than the sum of deaths by cancer and chronic lower respiratory disease[1]. Therefore, research aiming at identifying new therapeutic strategies to battle atherosclerosis pathology from an early stage is mandatory.

Atherosclerotic plaque formation is a chronic process which develops as a result of disturbed cholesterol regulation and immune system dysfunction[4]. Mast cells are immune components that have been described as important contributors in the progression of experimental mouse and human atherosclerosis[5]. The presence of mast cells within the plaques of human carotid arteries, was found to be a predictor of future cardiovascular events and furthermore, was associated with intraplaque hemorrhage levels, a characteristic of end-stage atherosclerosis[6]. Activation of mast cells in the plaque is suggested to lead to plaque destabilization[7], increasing thus the chances for an acute episode. However, the exact mechanism by which these cells get activated inside the plaque tissue has not been studied in detail.

In the work described throughout this thesis, we aimed to decipher the action of mast cells in experimental and human atherosclerosis. In **chapter 3** we determined the therapeutic potential of the $LPA_{1/3}$ antagonist, Ki16425, which was hypothesized to lead to inhibition of a recently described mast cell activator, termed lysophosphatidic acid[8]. **Chapters 4 and 5** examined the novel role of mast cell mediated antigen presentation in atherosclerosis. In **chapter 6**, by applying a translational approach, we determined the abundance and function of mast cells in human atherosclerotic plaques. Finally, in **chapter 7** we explored the influence of mast cells in atherosclerosis regression.

Mast cells act on multiple cardiovascular syndromes

The contribution of mast cells in the pathology of atherosclerosis is a subject that has gained considerable attention over the years. Upon contemplating the adverse effects that mast cell activation can elicit in other autoimmune pathologies, such as allergy[9] or rheumatoid arthritis[10], it is necessary to gain more information regarding their exact function in atherosclerotic disease. Therefore, during this thesis we aimed to unravel their mechanisms of action in atherosclerosis.

Chapter 2 offered a general overview on the implication of mast cells in various cardiovascular diseases (CVD). Even though the field of atherosclerosis has been actively investigating their function for the past 20 years, not much is known regarding other cardiovascular pathologies.

We reviewed the role of mast cells in diet induced obesity. Mast cells have been shown to infiltrate white adipose tissue[11] and contribute to local adipogenesis[12] and probably obesity. However, recently it was revealed that mast cells, through their secretion of leptin, can actually protect against obesity and diabetes, highlighting their dual-face actions[13]. This notion was strengthened by a report stating that mast cells can promote the formation of brown fat cells[14], which comprise the protective kind of adipocytes responsible for body temperature retention[15].

This double-edged nature of mast cells was also discussed in the case of myocardial infarction (MI). We conferred on their detrimental role in end-stage plaque rupture[16]. Their adverse nature in plaque destabilization was suggested in a new paper stating that mast cells are found at increased numbers in patients with stable and unstable coronary plaques, as well as in individuals who suffered from either an acute or chronic MI episode[17]. However, it seems that mast cells do not exert only harmful effects, since they are linked to neo-angiogenesis and healing of the myocardium after an acute MI episode[18,19]. Interestingly, a very recent report stated that following an MI incident, mast cells deriving from white adipose tissue can communicate with cardiomyocytes, increase their contractility and improve heart function[20].

The effect of mast cells on the pace of the myocardium may be particularly important in arrhythmia, as we also discussed in the second chapter. Indeed, the presence of mast cells has also been reported a few years ago in arrhythmic atrial fibrillation[21]. Heart rhythm-related dysfunctions are linked to atherosclerosis[22] and atrial fibrillation in particular shows an increasing predominance at present[1]. In fact, arrhythmia is widely understudied with respect to the contribution of the immune system. Interestingly, mast cells are a population that is fundamentally populating the heart at a normal state[23]. Although information available on the role of mast cells in arrhythmia is scarce, it seems that they act in a negative way[24,25]. Furthermore, a novel paradigm-shifting study revealed that also macrophages can influence the regulation of heart rhythm[26]. Mast cells are reported to interact with macrophages in other cardiovascular diseases such as atherosclerosis[27] and aortic aneurysms[28]. While this does not imply a causative relationship, it would be interesting to investigate in the future a possible interaction between tissue resident macrophages and cardiac mast cells in heart rhythm disorders.

It is therefore apparent that mast cells do not compose a cell type that is clear-

cut pro-inflammatory in cardiovascular syndromes. Examination of their function should be approached in a delicate fashion.

Modulation of immune responses in atherosclerosis through mast cells

Having in mind the above mentioned diverse abilities of mast cells, as well as the fact that atherosclerosis is the underlying pathology for the majority of CVDs, we aimed to experimentally elucidate their effects in atherosclerosis.

In **chapter 3** we examined the downstream effects of the established mast cell activator, lysophosphatidic acid (LPA)[29]. LPA is a bioactive lipid produced inside the atherosclerotic plaques where it has been shown to act in a proatherogenic manner[30,31]. LPA acts *via* its specific receptors $LPA_{1/3}$[32] on an array of immune cells[33] among which the mast cells[29,34]. For that reason, we inhibited the action of LPA by blocking the downstream signaling of receptors $LPA_{1/3}$, with the use of the small molecule Ki16425. $LPA_{1/3}$ inhibition had a significant impact on the systemic immune response, which resulted in a 40% reduction of the plaque burden, caused by a reduced macrophage content. The protective effect of Ki16425 was mainly attributed to the disruption of the CCR2-CCL2 infiltration axis, but we also observed an increase in the non-inflammatory monocyte content and the anti-inflammatory T_{REG} population. These effects were accompanied by a mild reduction in the LDL cholesterol levels. Overall, inhibition of $LPA_{1/3}$ appears to be an appealing therapeutic method to limit the progression of established atherosclerosis. Furthermore, our observations show that a shift of the systemic immune response, from pro-inflammatory to anti-inflammatory, in atherosclerosis can not only halt plaque progression, but can also influence cholesterol regulation. However, in this study we did not observe any significant mast cell contribution. Nonetheless, we still have strong indications that mast cells may also be affected by the LPA-signaling cascade. For example, mast cells have been reported to act also through additional LPA receptors, such as LPA_5, which in our study were still fully-functional[35]. Be that as it may, this did not seem to have any effect on the mast cell response we observed. The previously reported LPA action on the mast cells was introduced at advanced atherosclerosis stages[29], which was not the case in our experimental setup. The beneficial effects that we reported on the adaptive immune response suggest that $LPA_{1/3}$ inhibition may also be therapeutically relevant for a prolonged period. It would therefore be interesting to investigate this agent throughout a longer timeframe. In addition, the induction of T_{REG} cells opens the pathway to investigating this agent in atherosclerosis regression since T_{REG} cells are themselves an attractive therapeutic target[36,37].

The overall importance of the adaptive immune response, and particularly the one evoked by T cells is a known fact[38]. $CD4^+$ T cells, predominantly those of the T_{H1}

phenotype, are the most frequent inside atherosclerotic plaques[39]. In **chapter 4** we discussed a newly found direct interaction between mast cells and $CD4^+$ T cells in atherosclerosis. Knowing that mast cells are in close cross-talk with T cells in other inflammatory diseases[40,41], and based on novel reports on the role of mast cells as inducible antigen presenting cells[42,43], we aimed to study whether they interact with $CD4^+$ T cells in atherosclerosis. We reported that mast cells increase their antigen presentation capacity upon hyperlipidemia, by increasing their MHC-II expression in both experimental atherosclerosis, as well as inside human plaques. Through this crosstalk mast cells can present antigens directly to $CD4^+$ T cells, which, under the influence of a high-fat diet, are skewed towards a pro-atherogenic T_{H1} subtype. This is interesting, considering the fact that mast cells in allergies seem to favor a T_{H2} response instead[44]. It therefore seems that mast cells exert differential effects on T cells according to the local inflammatory milieu. Atherosclerosis, being a T_{H1} mediated disease, is probably favoring presentation of antigenic fragments that are affecting the T_{H1} subset. On the contrary, in allergies, which are mediated by T_{H2} cells, mast cells seem to enhance the T_{H2} response. It would be interesting to see if this mast cell-$CD4^+$ T cell crosstalk results in antibody production by the B cells. Screening for T_{H1}-linked antibody fragments is an attractive concept to explore further. Furthermore, we do not know what is the exact contribution of this pathway in atherosclerosis. It would be intriguing to see if abolishment of MHC-II, specifically from the surface of mast cells, can affect atherosclerosis progression *in vivo*. Also, we do not yet know which antigenic fragments are presented by mast cells to $CD4^+$ T-cells *via* MHC-II, in atherosclerosis. In the future, it may be appealing to scan the MHC-II epitopes of mast cells for lipid-specific antigenic presentation, as well as explore the pathway *via* which mast cells take up antigens from their surroundings. For instance, their uptake capacity could be investigated through differentially charged nanoparticles. Finally, a crucial question arises, on whether mast cells can take up lipids in a "foam-cell" fashion. Although we do not have indications that they possess classical uptake receptors, present on macrophages or dendritic cells, mast cells may take up lipids by other means and store them in forms other than neutral lipid accumulation.

The existence of a direct crosstalk between mast cells and the adaptive immune system was investigated also in **chapter 5,** where we observed a direct interaction with the NKT cell population, through CD1d-mediated lipid-specific presentation. Surprisingly, this pathway showed a protective effect in atherosclerosis development. This was an unexpected finding, considering that NKT cells, which in our study showed reduced activation, are reported to be proatherogenic, particularly upon engagement with CD1d[45]. However, we must remember that NKT cells can function as activators as well as inhibitors of the immune response, depending on the glycolipid that is presented to them[46,47]. In fact, it has been previously reported that NKT cells can negatively regulate

$CD4^+$ cells in atherosclerosis, in a fashion that matches our observations[48]. Furthermore, a protective role of NKT cells was also observed in other autoimmune syndromes where mast cells are known mediators, such as obesity[49] and rheumatoid arthritis[50]. Therefore, it seems that, while classical presentation of lipid antigens through CD1d present on APCs exacerbates atherosclerosis[51,52], antigen presentation through CD1d on the mast cell surface, exerts a protective effect. It is also interesting to observe that, although indirectly, mast cells again affect the $CD4^+$ T_{H1} cell response, as in the case of MHC-II mediated presentation. This interaction seems to be tightly balanced, and of importance, in atherosclerosis development; therefore, it requires further exploration.

The presentation capacity of human mast cells mentioned in the fourth chapter, was accompanied by a general phenotypic characterization of human mast cells as stated in **chapter 6**. While human intraplaque mast cells are the only immune cell type that was found to positively associate with future cardiovascular events[6], it is still not fully understood how these cells become activated within the atherosclerotic plaque. In this study we screened human intraplaque mast cells obtained from 22 carotid and femoral arteries, using for the first time the flow cytometry method. We confirmed immunohistochemical data from previous reports, which stated that the protease secretome of mast cells is highly heterogeneous[53], showing a differential cellular content of tryptase and chymase. Importantly, we detected high levels of activation by human intraplaque mast cells, based on their expression of protein CD63[54]. Of note, the majority of activated mast cells were IgE-sensitized, indicating that this pathway is the main activation mode of the human intraplaque mast cell population. This does not come as a surprise, since mast cells are mostly known for their classical degranulation potency upon antigen-sensitized IgE binding on their Fcε-receptor[55]. However, it may explain why serum IgE levels correlate in a positive manner with coronary artery disease[56]. This observation as mentioned above, has also been attributed to mast cells[6,17]. In addition, we noted that a minor fraction of these cells was activated in a non-IgE specific manner, thus strengthening the case of non-classical Fcε-receptor mediated activation of mast cells. It has been proven that mast cells in cardiovascular diseases can get activated by means other than FcεR, such as through TLRs[57], complement receptors[57] or neuropeptide receptors[58]. However, we did not have an indication on the proportion of these alternative activation pathways within the atherosclerotic plaque. The adverse effects that this seemingly small fraction of mast cells can exert in atherosclerosis progression, like in the case of substance P which is linked to intraplaque hemorrhage levels[59], indicates the magnitude of power that these cells possess in atherosclerosis. The above, therefore, states how important it is to consider therapeutic possibilities that target mast cells.

In **chapter 7**, we explored the therapeutic potential that arises from the

sheer absence of mast cells in atherosclerosis regression conditions. Specifically, we conditionally depleted mast cells, at a systemic level, upon altering the diet content from high-fat to normal chow. In our experimental setup we did not observe any reduction in the atherosclerotic plaque size; which indicates that depletion of mast cells after an ongoing inflammatory cascade, does not have the intensity to reduce the plaque volume. We did detect, however, a reduced neutrophil intraplaque influx, in the absence of mast cells. This effect can be explained by the fact that mast cells induce neutrophil infiltration into the plaque[60]. Interestingly, even though there was no effect detected in overall plaque burden, mast cell depletion affected the collagen content of the plaque in a negative manner. This was unexpected, since mast cells are negatively associated with collagen deposition inside atherosclerotic plaques, in a chymase specific action[61]. Yet, tryptase release has been reported to induce collagen synthesis in a renal fibrosis model[62], indicating again the complex manner by which these cells act.

Overall, the observations stated in this thesis suggest that when it comes to intervention of the mast cell action, we should not aim solely on their abolition as they do not appear to possess only harmful effects, but are rather fine tuners of the overall immune response.

Future perspectives

The research described in this thesis paves the way for exploring the therapeutic potential of targeting mast cells in atherosclerosis. Evidently, this cell type, as most immune cells, shows remarkable plasticity. As such, mast cells require refined targeting of specific pathways that would inhibit their negative actions, while retaining or even enhancing their positive effects.

In the case of atherosclerosis, mast cells are, for the most part damaging, when it comes to disease progression; particularly, through their FcεR mediated activation. This specific response is a promising therapeutic point, mainly due to the already commercially available agents that target its ligand, IgE[63]. Specifically, IgE has been found to circulate at high levels in the serum of patients suffering from an acute cardiovascular syndrome[64]. In addition, IgE levels independently correlate with the severity of coronary syndromes[56]. Here we reported that IgE can penetrate the endothelial barrier, accumulate inside the atherosclerotic plaque and bind on the surface of mast cells, thus sensitizing them. The accumulation of IgE within the atherosclerotic plaque does not necessarily require the participation of an antigen. However, most of the intraplaque mast cells we examined were found to have IgE bound on their surface and to be at an activated state. It therefore seems, that they have undergone at least one cycle of degranulation in the area. A previous study has stated that, IgE is elevated upon myocardial infarction[65]

and the authors detected IgE fragments inside human atherosclerosis specimens. However, this intraplaque IgE was argued to act mainly on the inducible FcεRI of the macrophages, and not on intraplaque mast cells. Here we have provided proof that the intraplaque IgE is also activating mast cells in the plaque area. For this process to take place, the presence of an antigen is also necessary. As mentioned above, we do not know which antigenic fragment may bind on IgE. It may however be speculated that the antigen derives directly from the atherosclerotic plaque environment. Further research is needed on the antigen-mediated activation of mast cells within the plaques. Yet, upon considering the harmful effects linked to intraplaque mast cell activation[7,66], it is obvious that intervention on this pathway may prove beneficial for end-stage CVD related events. The IgE-antibody blocking agent omalizumab[67], which is already on the market and prescribed for allergic asthma cases[68,69], as well as for patients suffering from urticaria[70] and mastocytosis[71], could prove beneficial also in atherosclerosis. Indeed, we are eager to report that we have gained permission to study the effects of omalizumab in atherosclerosis patients.

A more crude method to battle the adverse effects of mast cell activation could be through the use of mast cell stabilizers[72], such as cromolyn[73] or tranilast[74]. These agents act on the mast cell mediated degranulation pathway, mainly by affecting ion exchange between the cell and its microenvironment[75,76]. Mast cell stabilization *via* this method has been proven in the past to be beneficial in experimental atherosclerosis studies[7,77]. Since in our experimental work we showed that human intraplaque mast cells are highly activated, using a mast cell stabilizer to eliminate their degranulation sounds appealing. However, there are two important aspects to keep in mind. The first one is that the mode of action that stabilizers exert is not strictly specific for mast cells. For example, tranilast is known to influence also vascular smooth muscle cells[78], an effect which could risk adverse effects. The second aspect that needs to be noted is that inhibition of the mast cell degranulation machinery exclusively will alter also the release of mediators that are not necessarily pro-inflammatory. For example, mast cells are a source of the anti-inflammatory cytokine IL-10[78], which is known to be athero-protective[79,80]. In fact, mast cells have been recently reported to act in an immunoregulatory manner in bone marrow[81] as well as in solid organ transplantation[82,83]. Therefore, blocking the release of the entire mast cell secretome may interfere with the healing properties that these cells possess, while reducing protection towards pathogen infections. Furthermore, it is important to remember that mast cell activation does not necessarily mean degranulation. Mast cells can secrete pro-inflammatory cytokines like IL-8, in means that do not include release of their pre-stored granules[84]. A similar mode of mast cell activation has been previously reported through IL-1, which results in release of IL-6 but does not involve degranulation[85]. Therefore, while most activation pathways lead to mast cell degranulation, there are means which do not elicit such an effect, but rather lead

to controlled cytokine release. One additional example is the TLR activation pathway which results in increased cytokine production but not mast cell degranulation[86]. As mentioned before, oxLDL activates mast cells through TLR4[57]. This states how different the downstream mast cells action is inside the atherosclerotic plaque. According to our observations, approximately 20% of human intraplaque mast cells get activated *via* pathways other than IgE, which may include cytokine release without degranulation. In addition, it is known that when a cell undergoes activation, the intracellular energy consumption, and therefore the mitochondrial machinery, can alter its metabolic status. In the case of mast cells, recent work has stated that IgE mediated activation induces both oxidative phosphorylation and glycolysis[87]. Considering the fact that oxLDL acts *via* the TLR pathway[57] which does not elicit energy consuming degranulation[86], it would be interesting to see if this atherosclerosis specific pathway evokes any change in the cell metabolism of mast cells. Overall, it is important to exactly refine these differential activation pathways in the future, since distinct activators possibly lead to the release of different mediators, in both quality and quantity. After all it is the prevalence of the net immune effect that shapes disease progression.

As we demonstrated, mast cells possess also immunoregulatory abilities that seem to surpass the classical view of a cell that goes inside the atherosclerotic plaque to get activated, degranulate and inflict damage. The inducible antigenic presentation capacity of mast cells we mentioned, is an interesting target for therapeutic development. In particular, MHC-II expression on foam cells was reported to increase upon oxLDL uptake, which triggered an autophagy mediated pathway[88]. Mast cell degranulation is also reportedly mediated by autophagy signals, in a mechanism that is separate from their cytokine secretion machinery[89]. Interestingly, MHC-II induction on the mast cells is stated to increase in the presence of cytokine IL-33[90], a signal that also activates autophagy pathways[91]. It may therefore be that mast cell autophagy-mediated effects are also taking place in atherosclerosis, and affect the expression of MHC-II on the mast cell surface. Further exploration of such a pathway could be of importance in understanding the mast cell presentation capacity. Additionally, a past report indicated that MHC-II is stored in the intracellular mast cell compartment and fuses with the membrane upon degranulation[92]. Investigating whether disruption of this mechanism could reduce the established crosstalk between mast cells and $CD4^+$ T_{H1} cells could be helpful in a therapeutic context. Here we should also mention that mast cells do not seem to affect the adaptive immune system only *via* a direct action on the $CD4^+$ T cells, but can do so indirectly as well. There are studies that explore the exchange of antigenic content between mast cells and dendritic cells[93]. This can happen both through intracellular bridges[93], but it may also happen *via* the secretion of exosomes[94]. Neither the exosomal release by mast cells, nor the antigen-induced crosstalk between mast cells and DCs has been explored in atherosclerosis. However, in our study, we did see that more mast

cells infiltrate the para-aortic lymph nodes upon hyperlipidemia; particularly in the area where DCs reside. The accumulation of mast cells in the LNs upon atherosclerosis progression is on its own an additional pathway to explore. Are they for instance travelling there through the lymphatics? Are they able to migrate from one tissue to the other, carrying along tissue specific antigens? Or are they newly-maturated mast cells deriving from bone marrow progenitors? Answering these questions may provide evidence towards new therapeutic interventions.

An additional pathway that needs further exploration is the epigenetic pressure exerted on the mast cells, inside an inflammatory tissue. The final mast cell maturation step takes place within the tissue[94] in a multitude of organs where mast cells reside. This suggests that mast cells are a highly diverse population with different properties, depending on their place of residence. A recent study screened the transcriptome of mast cells collected from various tissues and confirmed this exact notion[95]. Mast cells indeed appeared to be highly sensitive depending on their surroundings. This raises a question regarding tissues that are characterized by chronic inflammatory pressure, such as the atherosclerotic plaque. Is the local environmental pressure able to alter the mast cell properties at a genetic level? Very recent pioneering work has in fact stated that mast cell tryptase can elicit epigenetic changes on the mast cell genome, through histone truncation[96]. This epigenetic effect is enhanced through time and affects the cellular identity of mast cells, which begin to show similarities with macrophages. It would be intriguing to examine if such a pathway exists also inside the atherosclerotic plaque. After all, epigenetic changes have gained recent attention in atherosclerosis[96].

In conclusion, the above work states that it is important to target specific pathways involved in differential mast cell actions. This is of particular clinical importance since these cells are not only crucial in inflammatory conditions[97], but are also the first responders in infections[98] and potent immune regulators in cancer[99]. After all it is their balancing nature that makes mast cells a fascinating cell type in atherosclerosis, worthy to be explored in depth.

Lastly, it is necessary to mention the therapeutic potential that is offered in atherosclerosis, by targeting the overall immune response. As shown by our work on the LPA-receptor inhibition, systemic modulation of the immune response can efficiently hamper atherosclerosis progression. At this moment, pre-clinical atherosclerosis is being pharmacologically treated with the use of statins[100]. Originally, the benefits of statin use were appointed to the lowering of non-HDL cholesterol alone. However, it was the combination with lower inflammatory burden that resulted in atherosclerotic plaque stabilization[100]. Yet up to now it has not been possible to reduce the plaque burden of an already established human plaque[101]. A novel approach for the reduction of LDL

has been put to practice very recently. Antibody-mediated inhibition of the expression of the hepatic protein PCSK9 is reported to lower LDL levels by 50%, showing very promising potential[102]. However, long term assessment on this type of medication is not possible at the moment. Atherosclerosis research has been actively focusing on means to achieve plaque regression, with experimental models spanning from stimulation of reverse cholesterol transport by means of micro-RNA antagonism[103], to inhibition of immune pathways, in combination with low-lipid diets[104,105]. Lastly, about two months ago, the large scale CANTOS trial, which introduced the inhibition of the inflammatory cytokine IL-1β, using the antibody canakinumab, met its primary clinical endpoint[106]. The results stated that patients treated with canakinumab showed a significant reduction in the risk of developing secondary cardiac events[107]. This anti-inflammatory therapeutic method is a scientific breakthrough comparable to the discovery of statins.

Ultimately, treating atherosclerosis is the challenge of the near future. A challenge that the scientific field has long accepted, in a duel that will be won. *Et lux in tenebris lucet*.

Reference list:

1. Benjamin, E. J. *et al.* Heart Disease and Stroke Statistics-2017 Update: A Report From the American Heart Association. *Circulation* **135,** e146–e603 (2017).
2. Vital statistics data - US Centers for Disease Control and Prevention. Available at: http://www.cdc.gov/nchs/data_access/vitalstatsonline.htm.
3. European Cardiovascular Disease Statistics - 2017. Available at: http://www.ehnheart.org/cvd-statistics.html.
4. Usman, A., Ribatti, D., Sadat, U. & Gillard, J. H. From Lipid Retention to Immune-Mediate Inflammation and Associated Angiogenesis in the Pathogenesis of Atherosclerosis. *J. Atheroscler. Thromb.* **22,** 739–749 (2015).
5. Shi, G.-P., Bot, I. & Kovanen, P. T. Mast cells in human and experimental cardiometabolic diseases. *Nat. Rev. Cardiol.* **12,** 643–658 (2015).
6. Willems, S. *et al.* Mast cells in human carotid atherosclerotic plaques are associated with intraplaque microvessel density and the occurrence of future cardiovascular events. *Eur. Heart J.* **34,** 3699–3706 (2013).
7. Bot, I. *et al.* Perivascular mast cells promote atherogenesis and induce plaque destabilization in apolipoprotein E-deficient mice. *Circulation* **115,** 2516–2525 (2007).
8. Bot, M. *et al.* Lysophosphatidic acid triggers mast cell-driven atherosclerotic plaque destabilization by increasing vascular inflammation. *J. Lipid Res.* **54,** 1265–74 (2013).
9. Saluja, R., Khan, M., Church, M. K. & Maurer, M. The role of IL-33 and mast cells in allergy and inflammation. *Clin. Transl. Allergy* **5,** 33 (2015).
10. Maruotti, N., Crivellato, E., Cantatore, F. P., Vacca, A. & Ribatti, D. Mast cells in rheumatoid arthritis. *Clin. Rheumatol.* **26,** 1–4 (2007).
11. Liu, J. *et al.* Genetic deficiency and pharmacological stabilization of mast cells reduce diet-induced obesity and diabetes in mice. *Nat. Med.* **15,** 940–945 (2009).
12. Tanaka, A., Nomura, Y., Matsuda, A., Ohmori, K. & Matsuda, H. Mast cells function as an alternative modulator of adipogenesis through 15-deoxy-delta-12, 14-prostaglandin J2. *Am. J. Physiol. Cell Physiol.* **301,** C1360-7 (2011).
13. Zhou, Y. *et al.* Leptin Deficiency Shifts Mast Cells toward Anti-Inflammatory Actions and Protects Mice from Obesity and Diabetes by Polarizing M2 Macrophages. *Cell Metab.* **22,** 1045–1058 (2015).
14. Finlin, B. S. *et al.* Mast Cells Promote Seasonal White Adipose Beiging in Humans. *Diabetes* **66,** 1237–1246 (2017).
15. Bartelt, A. & Heeren, J. Adipose tissue browning and metabolic health. *Nat. Rev. Endocrinol.* **10,** 24–36 (2014).
16. Laine, P. *et al.* Association Between Myocardial Infarction and the Mast Cells in the Adventitia of the Infarct-Related Coronary Artery. *Circulation* **99,** 361–369 (1999).
17. Kupreishvili, K. *et al.* Mast cells are increased in the media of coronary lesions in patients with myocardial infarction and may favor atherosclerotic plaque instability. *J. Cardiol.* **69,** 548–554 (2017).
18. Somasundaram, P. *et al.* Mast cell tryptase may modulate endothelial cell phenotype in healing myocardial infarcts. *J. Pathol.* **205,** 102–111 (2005).
19. Kwon, J. S. *et al.* The novel role of mast cells in the microenvironment of acute myocardial infarction. *J. Mol. Cell. Cardiol.* **50,** 814–825 (2011).
20. Ngkelo, A. *et al.* Mast cells regulate myofilament calcium sensitization and heart function after myocardial infarction. *J. Exp. Med.* **213,** 1353–1374 (2016).
21. Liao, C. *et al.* Cardiac mast cells cause atrial fibrillation through PDGF-A-mediated fibrosis in pressure-overloaded mouse hearts. *J. Clin. Invest.* **120,** 242–253 (2010).

22. De Jesus, N. M. *et al.* Atherosclerosis exacerbates arrhythmia following myocardial infarction: Role of myocardial inflammation. *Heart Rhythm* **12,** 169–178 (2015).
23. Rakusan, K., Sarkar, K., Turek, Z. & Wicker, P. Mast cells in the rat heart during normal growth and in cardiac hypertrophy. *Circ. Res.* **66,** 511–516 (1990).
24. Morrey, C. *et al.* Interaction between sensory C-fibers and cardiac mast cells in ischemia/reperfusion: activation of a local renin-angiotensin system culminating in severe arrhythmic dysfunction. *J. Pharmacol. Exp. Ther.* **335,** 76–84 (2010).
25. Koda, K. *et al.* Aldehyde dehydrogenase activation prevents reperfusion arrhythmias by inhibiting local renin release from cardiac mast cells. *Circulation* **122,** 771–781 (2010).
26. Hulsmans, M. *et al.* Macrophages Facilitate Electrical Conduction in the Heart. *Cell* **169,** 510–522.e20 (2017).
27. Yeong, P., Ning, Y., Xu, Y., Li, X. & Yin, L. Tryptase promotes human monocyte-derived macrophage foam cell formation by suppressing LXRalpha activation. *Biochim. Biophys. Acta* **1801,** 567–576 (2010).
28. Wang, J. *et al.* IgE actions on CD4+ T cells, mast cells, and macrophages participate in the pathogenesis of experimental abdominal aortic aneurysms. *EMBO Mol. Med.* **6,** 952–969 (2014).
29. Bot, M. *et al.* Lysophosphatidic acid triggers mast cell-driven atherosclerotic plaque destabilization by increasing vascular inflammation. *J. Lipid Res.* **54,** 1265–74 (2013).
30. Bot, M. *et al.* Atherosclerotic lesion progression changes lysophosphatidic acid homeostasis to favor its accumulation. *Am. J. Pathol.* **176,** 3073–3084 (2010).
31. Schober, A. & Siess, W. Lysophosphatidic acid in atherosclerotic diseases. *Br. J. Pharmacol.* **167,** 465–82 (2012).
32. Fukushima, N. & Chun, J. The LPA receptors. *Prostaglandins Other Lipid Mediat.* **64,** 21–32 (2001).
33. Yung, Y. C., Stoddard, N. C. & Chun, J. *LPA receptor signaling: pharmacology, physiology, and pathophysiology. Journal of lipid research* **55,** (2014).
34. Bagga, S. *et al.* Lysophosphatidic acid accelerates the development of human mast cells. *Blood* **104,** 4080–7 (2004).
35. Lundequist, A. & Boyce, J. A. LPA5 is abundantly expressed by human mast cells and important for lysophosphatidic acid induced MIP-1beta release. *PLoS One* **6,** e18192 (2011).
36. Foks, A. C., Lichtman, A. H. & Kuiper, J. Treating atherosclerosis with regulatory T cells. *Arterioscler. Thromb. Vasc. Biol.* **35,** 280–287 (2015).
37. Foks, A. C. *et al.* Differential effects of regulatory T cells on the initiation and regression of atherosclerosis. *Atherosclerosis* **218,** 53–60 (2011).
38. Tse, K., Tse, H., Sidney, J., Sette, A. & Ley, K. T cells in atherosclerosis. *Int. Immunol.* **25,** 615–622 (2013).
39. Jonasson, L., Holm, J., Skalli, O., Bondjers, G. & Hansson, G. K. Regional accumulations of T cells, macrophages, and smooth muscle cells in the human atherosclerotic plaque. *Arteriosclerosis* **6,** 131–138 (1986).
40. Gaudenzio, N., Laurent, C., Valitutti, S. & Espinosa, E. Human mast cells drive memory CD4+ T cells toward an inflammatory IL-22+ phenotype. *J. Allergy Clin. Immunol.* **131,** 1400–7.e11 (2013).
41. Sehra, S. *et al.* TH9 cells are required for tissue mast cell accumulation during allergic inflammation. *J. Allergy Clin. Immunol.* **136,** 433–40.e1 (2015).
42. Suurmond, J. *et al.* Communication between human mast cells and CD4(+) T cells through antigen-dependent interactions. *Eur. J. Immunol.* **43,** 1758–1768 (2013).
43. Kambayashi, T. & Laufer, T. M. Atypical MHC class II-expressing antigen-presenting cells:

can anything replace a dendritic cell? *Nat. Rev. Immunol.* **14,** 719–30 (2014).

44. Chai, O. H., Han, E.-H., Lee, H.-K. & Song, C. H. Mast cells play a key role in Th2 cytokine-dependent asthma model through production of adhesion molecules by liberation of TNF-alpha. *Exp. Mol. Med.* **43,** 35–43 (2011).
45. Tupin, E. *et al.* CD1d-dependent activation of NKT cells aggravates atherosclerosis. *J. Exp. Med.* **199,** 417–422 (2004).
46. van Puijvelde, G. H. M. & Kuiper, J. NKT cells in cardiovascular diseases. *Eur. J. Pharmacol.* (2017). doi:10.1016/j.ejphar.2017.03.052
47. Godfrey, D. I. & Kronenberg, M. Going both ways: immune regulation via CD1d-dependent NKT cells. *J. Clin. Invest.* **114,** 1379–1388 (2004).
48. Miyazaki, Y. *et al.* Effect of high fat diet on NKT cell function and NKT cell-mediated regulation of Th1 responses. *Scand. J. Immunol.* **67,** 230–237 (2008).
49. Huh, J. Y. *et al.* Deletion of CD1d in adipocytes aggravates adipose tissue inflammation and insulin resistance in obesity. *Diabetes* **66,** 835–847 (2017).
50. Teige, A. *et al.* CD1d-dependent NKT cells play a protective role in acute and chronic arthritis models by ameliorating antigen-specific Th1 responses. *J. Immunol.* **185,** 345–56 (2010).
51. Nakai, Y. *et al.* Natural killer T cells accelerate atherogenesis in mice. *Blood* **104,** 2051–2059 (2004).
52. Li, Y. *et al.* A CD1d-dependent lipid antagonist to NKT cells ameliorates atherosclerosis in ApoE-/- mice by reducing lesion necrosis and inflammation. *Cardiovasc. Res.* **109,** 305–317 (2016).
53. Kaartinen, M., Penttila, A. & Kovanen, P. T. Mast cells of two types differing in neutral protease composition in the human aortic intima. Demonstration of tryptase- and tryptase/chymase-containing mast cells in normal intimas, fatty streaks, and the shoulder region of atheromas. *Arterioscler. Thromb. a J. Vasc. Biol.* **14,** 966–972 (1994).
54. Kraft, S. *et al.* The tetraspanin CD63 is required for efficient IgE-mediated mast cell degranulation and anaphylaxis. *J. Immunol.* **191,** 2871–2878 (2013).
55. Burd, P. R. *et al.* Interleukin 3-dependent and -independent mast cells stimulated with IgE and antigen express multiple cytokines. *J. Exp. Med.* **170,** 245–257 (1989).
56. Guo, X. *et al.* Serum IgE levels are associated with coronary artery disease severity. *Atherosclerosis* **251,** 355–360 (2016).
57. Meng, Z. *et al.* Oxidized low-density lipoprotein induces inflammatory responses in cultured human mast cells via Toll-like receptor 4. *Cell. Physiol. Biochem.* **31,** 842–53 (2013).
58. Lagraauw, H. M. *et al.* Vascular neuropeptide Y contributes to atherosclerotic plaque progression and perivascular mast cell activation. *Atherosclerosis* **235,** 196–203 (2014).
59. Bot, I. *et al.* The neuropeptide substance P mediates adventitial mast cell activation and induces intraplaque hemorrhage in advanced atherosclerosis. *Circ. Res.* **106,** 89–92 (2010).
60. Wezel, A. *et al.* Mast cells mediate neutrophil recruitment during atherosclerotic plaque progression. *Atherosclerosis* **241,** 289–296 (2015).
61. Bot, I. *et al.* Mast cell chymase inhibition reduces atherosclerotic plaque progression and improves plaque stability in ApoE-/- mice. *Cardiovasc. Res.* **89,** 244–252 (2011).
62. Kondo, S. *et al.* Role of mast cell tryptase in renal interstitial fibrosis. *J. Am. Soc. Nephrol.* **12,** 1668–1676 (2001).
63. Wright, J. D. *et al.* Structural and Physical Basis for Anti-IgE Therapy. *Sci. Rep.* **5,** 11581 (2015).
64. Erdogan, O., Gul, C., Altun, a. & Ozbay, G. Increased Immunoglobulin E Response in Acute

Coronary Syndromes. *Angiology* **54,** 73–79 (2003).

65. Wang, J. *et al.* IgE stimulates human and mouse arterial cell apoptosis and cytokine expression and promotes atherogenesis in Apoe-/- mice. *J. Clin. Invest.* **121,** 3564–3577 (2011).
66. Leskinen, M. J., Kovanen, P. T. & Lindstedt, K. A. Regulation of smooth muscle cell growth, function and death in vitro by activated mast cells--a potential mechanism for the weakening and rupture of atherosclerotic plaques. *Biochem. Pharmacol.* **66,** 1493–1498 (2003).
67. Kawakami, T. & Blank, U. From IgE to Omalizumab. *J. Immunol.* **197,** 4187–4192 (2016).
68. Pelaia, G. *et al.* Anti-IgE therapy with omalizumab for severe asthma: current concepts and potential developments. *Curr. Drug Targets* **16,** 171–178 (2015).
69. Humbert, M. *et al.* Omalizumab in asthma: an update on recent developments. *J. allergy Clin. Immunol. Pract.* **2,** 525–36.e1 (2014).
70. Sellitto, A. *et al.* Effects of omalizumab treatment on serum cytokine concentrations of atopic patients with chronic spontaneous urticaria: a preliminary report. *Eur. Ann. Allergy Clin. Immunol.* **49,** 171–175 (2017).
71. Sokol, K. C., Ghazi, A., Kelly, B. C. & Grant, J. A. Omalizumab as a desensitizing agent and treatment in mastocytosis: a review of the literature and case report. *J. allergy Clin. Immunol. Pract.* **2,** 266–270 (2014).
72. Finn, D. F. & Walsh, J. J. Twenty-first century mast cell stabilizers. *Br. J. Pharmacol.* **170,** 23–37 (2013).
73. Edwards, A. M. & Norris, A. A. Cromoglycate and asthma. *Lancet (London, England)* **343,** 426 (1994).
74. Baba, A. *et al.* Anti-Allergic Drugs Tranilast and Ketotifen Dose-Dependently Exert Mast Cell-Stabilizing Properties. *Cell. Physiol. Biochem.* **38,** 15–27 (2016).
75. Alton, E. W. & Norris, A. A. Chloride transport and the actions of nedocromil sodium and cromolyn sodium in asthma. *J. Allergy Clin. Immunol.* **98,** S102-5-6 (1996).
76. Garcia Mesa, M. New approach to the mechanism of antiasthmatic action of Tranilast. *Allergol. Immunopathol. (Madr).* **18,** 53–56 (1990).
77. Saiura, A., Sata, M., Hirata, Y., Nagai, R. & Makuuchi, M. Tranilast inhibits transplant-associated coronary arteriosclerosis in a murine model of cardiac transplantation. *Eur. J. Pharmacol.* **433,** 163–168 (2001).
78. Nie, L., Mogami, H., Kanzaki, M., Shibata, H. & Kojima, I. Blockade of DNA synthesis induced by platelet-derived growth factor by tranilast, an inhibitor of calcium entry, in vascular smooth muscle cells. *Mol. Pharmacol.* **50,** 763–769 (1996).
79. Han, X. & Boisvert, W. A. Interleukin-10 protects against atherosclerosis by modulating multiple atherogenic macrophage function. *Thromb. Haemost.* **113,** 505–512 (2015).
80. McCarthy, C. *et al.* IL-10 mediates the immunoregulatory response in conjugated linoleic acid-induced regression of atherosclerosis. *FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol.* **27,** 499–510 (2013).
81. Leveson-Gower, D. B. *et al.* Mast cells suppress murine GVHD in a mechanism independent of CD4+CD25+ regulatory T cells. *Blood* **122,** 3659–3665 (2013).
82. Jungraithmayr, W. The putative role of mast cells in lung transplantation. *Am. J. Transplant* **15,** 594–600 (2015).
83. Nakano, T. *et al.* Immunological and regenerative aspects of hepatic mast cells in liver allograft rejection and tolerance. *PLoS One* **7,** e37202 (2012).
84. Lin, T. J., Issekutz, T. B. & Marshall, J. S. Human mast cells transmigrate through human umbilical vein endothelial monolayers and selectively produce IL-8 in response to stromal cell-derived factor-1 alpha. *J. Immunol.* **165,** 211–220 (2000).

85. Conti, P. *et al.* Mast cells emerge as mediators of atherosclerosis: Special emphasis on IL-37 inhibition. *Tissue Cell* **49,** 393–400 (2017).

86. Suurmond, J., Dorjee, A. L., Knol, E. F., Huizinga, T. W. J. & Toes, R. E. M. Differential TLR-induced cytokine production by human mast cells is amplified by FcvarepsilonRI triggering. *Clin. Exp. Allergy* **45,** 788–796 (2015).

87. Phong, B., Avery, L., Menk, A. V, Delgoffe, G. M. & Kane, L. P. Cutting Edge: Murine Mast Cells Rapidly Modulate Metabolic Pathways Essential for Distinct Effector Functions. *J. Immunol.* **198,** 640–644 (2017).

88. Choi, S.-H. *et al.* SYK regulates macrophage MHC-II expression via activation of autophagy in response to oxidized LDL. *Autophagy* **11,** 785–795 (2015).

89. Nakano, H. & Ushio, H. An unexpected role for autophagy in degranulation of mast cells. *Autophagy* **7,** 657–659 (2011).

90. Ito, T. *et al.* IL-33 promotes MHC class II expression in murine mast cells. *Immunity, Inflamm. Dis.* **3,** 196–208 (2015).

91. Wu, J. *et al.* IL-33 is required for disposal of unnecessary cells during ovarian atresia through regulation of autophagy and macrophage migration. *J. Immunol.* **194,** 2140–2147 (2015).

92. Raposo, G. *et al.* Accumulation of major histocompatibility complex class II molecules in mast cell secretory granules and their release upon degranulation. *Mol. Biol. Cell* **8,** 2631–2645 (1997).

93. Carroll-Portillo, A. *et al.* Mast cells and dendritic cells form synapses that facilitate antigen transfer for T cell activation. *J. Cell Biol.* **210,** 851–864 (2015).

94. Skokos, D. *et al.* Mast cell-derived exosomes induce phenotypic and functional maturation of dendritic cells and elicit specific immune responses in vivo. *J. Immunol.* **170,** 3037–3045 (2003).

95. Dwyer, D. F., Barrett, N. A. & Austen, K. F. Expression profiling of constitutive mast cells reveals a unique identity within the immune system. *Nat. Immunol.* **17,** 878–887 (2016).

96. Melo, F. R. *et al.* Tryptase-catalyzed core histone truncation: A novel epigenetic regulatory mechanism in mast cells. *J. Allergy Clin. Immunol.* **140,** 474–485 (2017).

97. Frenzel, L. & Hermine, O. Mast cells and inflammation. *Joint. Bone. Spine* **80,** 141–145 (2013).

98. Voehringer, D. Protective and pathological roles of mast cells and basophils. *Nat. Rev. Immunol.* **13,** 362–375 (2013).

99. Khazaie, K. *et al.* The significant role of mast cells in cancer. *Cancer Metastasis Rev.* **30,** 45–60 (2011).

100. Nissen, S. E. Effect of intensive lipid lowering on progression of coronary atherosclerosis: evidence for an early benefit from the Reversal of Atherosclerosis with Aggressive Lipid Lowering (REVERSAL) trial. *Am. J. Cardiol.* **96,** 61F–68F (2005).

101. Nissen, S. E. *et al.* Effect of very high-intensity statin therapy on regression of coronary atherosclerosis: the ASTEROID trial. *JAMA* **295,** 1556–1565 (2006).

102. Whayne, T. F. J. PCSK9 inhibitors in the current management of atherosclerosis. *Arch. Cardiol. Mex.* **87,** 43–48 (2017).

103. Rayner, K. J. *et al.* Antagonism of miR-33 in mice promotes reverse cholesterol transport and regression of atherosclerosis. *J. Clin. Invest.* **121,** 2921–2931 (2011).

104. Foks, A. C. *et al.* Interruption of the OX40 – OX40 Ligand Pathway in LDL Receptor – Deficient Mice Causes Regression of Atherosclerosis. (2013). doi:10.4049/jimmunol.1200708

105. Kita, T. *et al.* Regression of atherosclerosis with anti-CD3 antibody via augmenting a regulatory T-cell response in mice. *Cardiovasc. Res.* **102,** 107–117 (2014).

106. Ridker, P. M., Thuren, T., Zalewski, A. & Libby, P. Interleukin-1beta inhibition and the

prevention of recurrent cardiovascular events: rationale and design of the Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS). *Am. Heart J.* **162,** 597–605 (2011).

107. Ridker, P. M. *et al.* Antiinflammatory Therapy with Canakinumab for Atherosclerotic Disease. *N. Engl. J. Med.* (2017). doi:10.1056/NEJMoa1707914

Dutch summary

Nederlandse samenvatting

Slagaderverkalking oftewel atherosclerose is de belangrijkste onderliggende oorzaak van acute cardiovasculaire syndromen zoals een hartinfarct of beroerte[1]. Hart- en vaatziekten waren in 2014 verantwoordelijk voor 1 op de 3 doden onder volwassenen in de Verenigde Staten, waarbij gemiddeld 1 sterfgeval per 40 seconden te betreuren viel[2]. In Europa zijn hart- en vaatziekten verantwoordelijk voor 45% van alle doden volgens de huidige statistieken van 2017[3]. Hoewel het aantal doden door kanker de afgelopen jaren sterk gestegen is, is de mortaliteit door hart- en vaatziekten vandaag de dag hoger dan het aantal doden door kanker en chronische luchtwegaandoeningen bij elkaar[1]. Daarom is het onderzoek naar nieuwe therapeutische strategieën, die de ontwikkeling van atherosclerose vanaf een vroeg stadium tegengaan, noodzakelijk.

De ontwikkeling van een atherosclerotische plaque is een chronisch proces, dat voortkomt uit een verstoorde lipidenhuishouding en dysfunctie van het immuunsysteem[4]. De mestcel is een cel behorende tot dit immuunsysteem, die beschreven is betrokken te zijn bij de progressie van atherosclerose in zowel muizen als mensen[5]. Het aantal mestcellen, dat aanwezig is plaques in de halsslagader van patiënten, bleek een voorspellende waarde te hebben voor het krijgen van een toekomstige acute cardiovasculaire aandoening, en was ook geassocieerd met de aanwezigheid van intraplaque bloedingen, een karakteristiek van plaque instabiliteit[6]. Activatie van de mestcel in de plaque kan leiden tot plaque destabilisatie[7], wat de kans op het krijgen van bijvoorbeeld een hartinfarct kan verhogen. De exacte mechanismen, die kunnen leiden tot mestcelactivatie in de plaque, zijn tot op heden echter niet tot in detail onderzocht.

In dit proefschrift hebben we de bijdrage van mestcellen aan de ontwikkeling van zowel experimentele als humane atherosclerose onderzocht.

In **hoofdstuk 3** hebben we de therapeutische potentie van de LPA_{1-3} receptor antagonist Ki15425, een stof die de activatie van mestcellen door lysofosfatidaat (lysophosphatidic acid, LPA) kan remmen, onderzocht[8]. In **hoofdstukken 4 en 5** is de tot op heden onbekende bijdrage van mestcel-gemedieerde antigen presentatie aan atherosclerose bekeken. In **hoofdstuk 6** hebben we bepaald in welke mate mestcellen aanwezig zijn in humane atherosclerotische plaques, en daarnaast de functie van de mestcel in deze plaques onderzocht om de mogelijke translatie van onze *in vivo* data naar de patient in kaart te brengen. Tenslotte is in **hoofdstuk 7** de bijdrage van de mestcel aan atherosclerotische plaque regressie onderzocht.

Modulatie van de immuunrespons in atherosclerose door mestcellen

Het onderzoek naar de bijdrage van de mestcel aan de pathologie van atherosclerose heeft de laatste jaren in toenemende mate aandacht gekregen. Activatie van mestcellen kan nadelige effecten hebben bij verschillende immuun-gerelateerde ziekten zoals allergie[9] or reumatoïde artritis[10], en het is ook noodzakelijk om de exacte bijdrage van deze cel aan de ontwikkeling van atherosclerose te onderzoeken.

Hoofdstuk 2 van dit proefschrift geeft een algemeen overzicht van de effecten van de mestcel op hart- en vaatziekten. De effecten van de mestcel op de ontwikkeling van atherosclerose zijn de afgelopen 20 jaar onderzocht en dit onderzoek hebben we in dit hoofdstuk samengevat. Er is echter nog niet veel bekend over de effecten van de mestcel op ander cardiovasculaire pathologieën. In dit hoofdstuk hebben we ook studies naar de bijdrage van de mestcel aan dieet-geïnduceerde obesitas beschreven. Mestcellen kunnen infiltreren in het vetweefsel, en daar een bijdrage leveren aan adipogenese en obesitas. Recentelijk is echter aangetoond dat mestcellen door de secretie van leptine ook zouden kunnen beschermen tegen obesitas en diabetes, wat de tweeledige rol van de mestcel illustreert[13]. Dit werd nog eens bevestigd door een artikel waarin is beschreven dat mestcellen de vorming van bruin vet[14], een type vet dat betrokken is bij onze temperatuurhuishouding[15], kan bevorderen. Deze verschillende functies van de mestcel zijn ook van belang bij bijvoorbeeld een hartinfarct. Mestcellen zijn eerder aangetoond betrokken te zijn bij plaqueruptuur[16], en ook in een recent artikel bleek de mestcel in verhoogde aantallen aanwezig in patiënten met zowel stabiele als instabiele plaques in de kransslagader, en in patiënten die zijn getroffen door een hartinfarct[17]. Mestcellen hebben daar echter niet alleen nadelige effecten, aangezien deze cellen ook van belang zijn bij de vorming van nieuwe bloedvaten, en bij het genezen van het myocardium na een acuut infarct[18,19]. Zeer recentelijk is zelfs aangetoond dat mestcellen uit het vetweefsel kunnen communiceren met cardiomyocyten, en daarmee de contractiliteit van de cardiomyocyten en de hartfunctie kunnen bevorderen[20].

Mestcellen kunnen effecten hebben op de snelheid van het hartritme, wat uitermate van belang kan zijn bij aritmieën, zoals we ook beschrijven in het tweede hoofdstuk. Mestcellen zijn een aantal jaren geleden beschreven bij atriumfibrilleren[21], en verschillende hartritmestoornissen, en met name atriumfibrilleren, zijn geassocieerd met atherosclerose[1,22]. Aritmie in relatie tot het immuunsysteem is tot op heden nog niet tot in detail onderzocht. Hoewel de mestcel ook onder gezonde omstandigheden al aanwezig is in het hart[23], lijkt de bijdrage van de mestcel bij het ontstaan van hartritmestoornissen ongunstig[24,25]. Naast mestcellen kunnen ook macrofagen het hartritme beinvloeden[26]. Mestcellen kunnen een interactie aangaan met macrofagen in cardiovasculaire aandoeningen zoals atherosclerose[27] en bij de vorming van een

aneurysma[28]. Hoewel dit geen causaal verband bewijst, zou het zeer interessant zijn om de mogelijke interactie tussen weefselmacrofagen en mestcellen in het hart in hartritmestoornissen te onderzoeken.

Omdat mestcellen zeer verschillende effecten op het omliggende weefsel kunnen hebben, en omdat atherosclerose de onderliggende pathologie is van het merendeel van de hart- en vaatziekten, hebben wij als doel gesteld de bijdrage van de mestcel aan experimentele atherosclerose en de effecten op de ontstekingsreactie te onderzoeken.

In **hoofdstuk 3** hebben we de remming van LPA-gemedieerde mestcelactivatie[29] op de ontwikkeling van atherosclerose onderzocht. LPA is een bioactief lipide, dat accumuleert in de atherosclerotische plaque, waar het bij kan dragen aan de progressie van atherosclerose[30,31]. LPA bindt aan specifieke $LPA_{1/3}$ receptoren[32], die zich op verscheidene immuuncellen[33] waaronder de mestcel[29,34] bevinden. Daarom hebben we in deze studie de effecten van LPA geremd door de $LPA_{1/3}$ receptoren te blokkeren met de chemische stof Ki16425. $LPA_{1/3}$ blokkade bleek een significant effect te hebben op de systemische immuunrespons en resulteerde in een 40% remming van plaquevorming, wat veroorzaakt werd door een verminderde hoeveelheid macrofagen in de plaque. Het beschermende effect van Ki16425 kwam door een verstoring van de CCR2-CCL2 interactie, maar we zagen ook een toename in de hoeveelheid anti-inflammatoire monocyten en regulatoire T cellen. Daarnaast observeerden we ook een lichte daling in de circulerende LDL cholesterol niveaus. Over het geheel bekeken lijkt de blokkade van $LPA_{1/3}$ een interessante therapeutische methode om de progressie van atherosclerose te remmen. Onze data laten zien dat blokkade van $LPA_{1/3}$ niet alleen een verschuiving van een pro- naar een anti-inflammatoire respons kan induceren en plaqueprogressie kan verminderen, maar ook de cholesterolniveaus kan reguleren. We konden in deze studie echter geen effecten op mestcellen aantonen. Desalniettemin zijn er nog steeds aanwijzingen dat LPA de mestcel kan beïnvloeden. Er zijn bijvoorbeeld andere LPA receptoren die door de mestcel tot expressie worden gebracht, die niet geblokkeerd werden in deze studie[35]. Daarnaast werden de effecten van LPA op mestcellen met name waargenomen in vergevorderde atherosclerose[29], een stadium dat we in deze studie niet bekeken hebben. Het gunstige effect dat wij hebben waargenomen op de verworven immuniteit suggereert dat blokkade van $LPA_{1/3}$ ook voor een langere periode effectief zou kunnen zijn. Het zou daarom zeer interessant zijn om deze de effecten van deze stof in een langdurige studie te bekijken De toename in het aantal regulatoire T cellen geeft daarnaast nog eens een extra aanleiding om $LPA_{1/3}$ blokkade nader te onderzoeken, bijvoorbeeld in plaque regressie, aangezien ook deze cellen een aantrekkelijk therapeutisch aangrijpingspunt zijn[36,37].

De betrokkenheid van de verworven immuunrespons, en voornamelijk de bijdrage van de T cellen, in atherosclerose is welbekend[38]. $CD4^+$ T cellen, en met name

de T_{H1} cellen, zijn in grote getalen aanwezig in de atherosclerotische plaque[39]. In **hoofdstuk 4** bediscussiëren we een nieuw gevonden interactie tussen mestcellen en $CD4^+$ T cellen in atherosclerose. Wetende dat mestcellen kunnen communiceren met T cellen in andere ontstekingsziekten[40,41], en gebaseerd om nieuwe studies waarbij de antigen presenterende functie van mestcellen wordt beschreven[42,43], hebben wij onderzocht of mestcellen een interactie aangaan met $CD4^+$ T cellen in atherosclerose. We beschrijven in dit hoofdstuk dat mestcellen de antigen presenterende capaciteit kunnen verhogen gedurende hyperlipidemie door de MHC-II expressie in zowel experimentele atherosclerose als in humane plaques te verhogen. Hierdoor kunnen mestcellen direct antigenen presenteren aan $CD4^+$ T cellen, die dan onder invloed van een hoog vet dieet differentiëren naar een pro-atherogeen T_{H1} subtype. Dit is interessant omdat mestcellen in allergie voornamelijk een T_{H2} respons bevorderen[44]. Mestcellen lijken daarom verschillende effecten te kunnen hebben op T cellen, afhankelijk van de ontsteking in het lokale milieu. Atherosclerose, als T_{H1} gemedieerde ziekte, zorgt waarschijnlijk voor presentatie van antigenen, die de T_{H1} subset beïnvloeden. In allergieën daarentegen lijken mestcellen een T_{H2} respons te bevorderen. Het zou daarom interessant zijn om te onderzoeken of deze mestcel-$CD4^+$ T cel interactie resulteert in antilichaamproductie door B cellen. Daarnaast kunnen we bekijken of specifieke depletie van MHC-II op de mestcel de progressie van atherosclerose *in vivo* kan beïnvloeden. Daarbij weten we nog niet welke antigenen via MHC-II gepresenteerd worden door mestcellen aan $CD4^+$ T-cellen in atherosclerose. In de toekomst zou het interessant kunnen zijn om MHC-II epitopen op mestcellen te screenen voor lipiden-specifieke antigenen, en daarnaast weten we nog niet via welk mechanisme antigenen door de mestcellen worden opgenomen uit de omgeving. Een laatste cruciale vraag is of mestcellen lipiden kunnen opnemen en opslaan als zogenaamde “foam-cellen”. Er zijn tot op heden geen aanwijzingen dat mestcellen klassieke opnamereceptoren hebben voor lipiden zoals het geval bij macrofagen en dendritische cellen, maar deze mestcellen zouden lipiden mogelijk kunnen opnemen via andere mechanismen, en vervolgens opslaan als neutrale lipiden.

In **hoofdstuk 5** hebben we de interactie tussen mestcellen en het verworven immuunsysteem in atherosclerose verder onderzocht door mestceldeficiente muizen te repopuleren met CD1d deficiënte of controle mestcellen. In deze studie hebben we een directe interactie tussen mestcellen en de NKT cel populatie gevonden via CD1d-gemedieerde presentatie van lipiden. Verrassend genoeg zagen we in deze studie een beschermend effect van mestcellen in atherosclerose. Dit was een onverwachte bevinding aangezien NKT cellen, die in deze studie verminderd geactiveerd bleken, in de literatuur als pro-atherogeen worden aangeduid, voornamelijk via presentatie van lipiden door CD1d[45]. NKT cellen kunnen echter zowel als activator als als remmer van de immuunrespons functioneren, afhankelijk van het glycolipide dat aan deze cellen

gepresenteerd wordt[46,47]. Het is zelfs aangetoond dat NKT cellen $CD4^+$ T cellen in atherosclerose kunnen remmen op een soortgelijk manier als wij in deze studie hebben geobserveerd[48]. Daarnaast kunnen NKT cellen een beschermende rol spelen in auto-immuunziekten zoals obesitas[49] en reumatoïde artritis[50], waarbij ook mestcelen een rol kunnen spelen. Antigen presentatie via CD1d op mestcellen lijkt daarom, in tegenstelling tot de klassieke presentatie van lipide antigenen via CD1d op antigen presenterende cellen die atherosclerose bevorderen[51,52], beschermend te werken. Hoewel het interessant is dat mestcellen ook in deze studie de $CD4^+$ T_{H1} cel respons beïnvloeden, alhoewel indirect, lijkt deze interactie sterk afhankelijk van de stimulans, en is er meer onderzoek nodig om de exacte bijdrage van deze mechanismen in atherosclerose te bevestigen.

De antigeen presenterende capaciteit van humane mestcellen genoemd in hoofdstuk 4 was een onderdeel van een algemene karakterisatie van humane mestcellen zoals beschreven in **hoofdstuk 6**. Hoewel de mestcel de enige immuuncel is waarvan de correlatie met toekomstige cardiovasculaire events is beschreven[6], is het activatiemechanisme van de mestcellen in de vergevorderde atherosclerotische plaque nog niet tot in detail bekend. In deze studie hebben we mestcellen uit 22 arteriën, verkregen van endarterectomie operaties (carotis en femoralis), geïsoleerd en gekarakteriseerd middels flow cytometry. We hebben met de verkregen flow cytometry data bestaande immunohistochemische data bevestigd, waarin is beschreven dat de mestcellen verschillende proteasen kunnen bevatten[53]. De mestcellen bleken heterogene hoeveelheden chymase en tryptase te produceren. Van belang was dat een groot aantal mestcellen in de plaque geactiveerd bleek, gebaseerd op de expressie van CD63 als marker voor mestcelactivatie[54]. Van deze geactiveerde mestcellen was de meerderheid gesensitiseerd met IgE, wat aangeeft dat dit een belangrijk mestcelactivatie mechanisme kan zijn in atherosclerose. Dit is niet verrassend, aangezien mestcellen bekend staan om de klassieke degranulatie via antigen-gesensitiseerde IgE binding op Fcε-receptoren[55], zoals bijvoorbeeld in allergieën. Dit zou ook kunnen verklaren waarom serum IgE niveaus positief correleren met de aanwezigheid van coronary artery disease (CAD)[56], wat ook geassocieeerd is met de aanwezigheid van mestcellen[6,17]. Er was daarnaast ook een kleinere fractie mestcellen, die was geactiveerd via mechanismen anders dan via IgE, wat aangeeft dat er meerdere mechanismen zijn waardoor mestcellen kunnen worden geactiveerd in de atherosclerotische plaque. Verschillende *in vivo* studies beschrijven dat mestcellen in hart- en vaatziekten kunnen worden geactiveerd via bijvoorbeeld TLRs[57], complement receptoren[57] of neuropeptide receptoren[58]. Tot op heden hebben we echter nog geen indicatie voor de bijdrage van deze alternatieve activatiemechanismen aan humane atherosclerotische plaque destabilisatie. De nadelige effecten echter die mestcellen kunnen hebben op plaquestabiliteit, zoals het induceren van intraplaque bloedingen[59], geven het belang aan van deze cellen in plaque

destabilisatie, en onderstrepen de potentie van therapieën die op mestcellen kunnen aangrijpen.

In **hoofdstuk 7** hebben we de therapeutische capaciteit van mestcellen in atherosclerotische plaque regressie onderzocht. In deze studie hebben mestcellen conditioneel gedepleteerd in de muizen, die al atherosclerose hadden ontwikkelend. Tegelijkertijd is ook het dieet gewisseld van een hoog vet dieet naar normaal knaagvoer. Met deze experimentele opzet hebben we geen effecten van mestceldepletie op de plaquegrootte waargenomen, wat aangeeft dat depletie van mestcellen op het moment dat er al een ontstekingsproces gaande is, geen bijdrage levert aan het reduceren van een bestaande atherosclerotische plaque. In deze studie bleek het aantal neutrofielen in de plaque wel sterk verlaagd na depletie van mestcellen, wat verklaard kan worden door de afwezigheid van chemotactische stoffen die een mestcel kan uitscheiden. Mestcellen kunnen door het uitscheiden van chemokines bij voorkeur neutrofielen naar de plaque recruiteren[60]. Ook bleek het collageengehalte in de plaque verlaagd door mestceldepletie, en dit was onverwacht aangezien mestcellen middels het uitscheiden van chymase[61] collagen in de plaque kunnen afbreken. Tryptase kan aan de andere kan collageensynthese induceren, zoals is gebleken in een nierfibrose model[62], wat de complexiteit van deze mestcellen in ziekteprocessen illustreert. Dit maakt mede duidelijk dat de mestcel geen celtype is wat eenduidig pro-inflammatoir is in hart- en vaatziekten en de functie van deze cel dient in detail per specifiek ziektebeeld te worden onderzocht. De data gegenereert in dit proefschrift illustreren dat we, in de zoektocht naar nieuwe therapeutische strategieen, niet enkel naar depletie of complete remming van mestcellen zouden moeten te kijken, aangezien de mestcel niet enkel nadelige effecten laat zien, maar ook als fine-tuner van de lokale immuunrespons kan optreden.

Op dit moment worden atherosclerose met name farmacologisch behandeld middels statines[63], die ontwikkeld zijn als geneedmiddel om het non-HDL cholesterol te verlagen. Statines bleken echter ook ontstekingsremmend te werken, en dit resulteerde in atherosclerotische plaque stabilisatie[63]. Een nieuwe aanpak voor het verlagen van LDL is recentelijk in de praktijk gebracht. De expressie van het levereiwit PCSK9 kan door middel van antilichaamtherapie verlaagd worden, wat uiteindelijk leidt tot een 50% verlaging van circulerende LDL niveaus, wat een zeer veelbelovende uitkomst is[65]. Het is tot op heden nog niet mogelijk om een bestaande atherosclerotische plaque te verminderen (plaque regressie)[64]. De lange-termijn effecten van deze therapie zijn nog niet bekend, maar wellicht zou regressie mogelijk zijn. Het induceren van plaque regressie zou een therapeutische doorbraak zijn, en het huidige onderzoek richt zich dan ook op het bereiken van plaque regressie. In dierexperimentele modellen worden bijvoorbeeld het stimuleren van reverse cholesterol transport door het antagoneren van specifieke micro-RNAs[66], of het remmen van immuunmechanismen in combinatie

met lipidenverlaging onderzocht[67,68]. Het zou ook interessant kunnen zijn om $LPA_{1/3}$ blokkade in de context van regressie te bestuderen.

Tenslotte heeft ongeveer twee maanden geleden de grote CANTOS trial, waarin remming van het proinfllammatoire cytokine IL-1β door het antilichaam canakinumab is onderzocht, het klinische eindpunt bereikt[69]. Patienten behandeld met canakinumab hadden een siginificante verlaging in het risico op het krijgen van een secundair cardiovasculair event[70]. Dit anti-inflammatoire geneesmiddel betekent een wetenschappelijke doorbraak, vergelijkbaar met de ontdekking van de statines, die de weg vrij maakt voor de ontwikkeling van nieuwe ontstekingsremmende geneesmiddelen tegen hart- en vaatziekten.

Referenties:

1. Benjamin, E. J. *et al.* Heart Disease and Stroke Statistics-2017 Update: A Report From the American Heart Association. *Circulation* **135,** e146–e603 (2017).
2. Vital statistics data - US Centers for Disease Control and Prevention. Available at: http://www.cdc.gov/nchs/data_access/vitalstatsonline.htm.
3. European Cardiovascular Disease Statistics - 2017. Available at: http://www.ehnheart.org/cvd-statistics.html.
4. Usman, A., Ribatti, D., Sadat, U. & Gillard, J. H. From Lipid Retention to Immune-Mediate Inflammation and Associated Angiogenesis in the Pathogenesis of Atherosclerosis. *J. Atheroscler. Thromb.* **22,** 739–749 (2015).
5. Shi, G.-P., Bot, I. & Kovanen, P. T. Mast cells in human and experimental cardiometabolic diseases. *Nat. Rev. Cardiol.* **12,** 643–658 (2015).
6. Willems, S. *et al.* Mast cells in human carotid atherosclerotic plaques are associated with intraplaque microvessel density and the occurrence of future cardiovascular events. *Eur. Heart J.* **34,** 3699–3706 (2013).
7. Bot, I. *et al.* Perivascular mast cells promote atherogenesis and induce plaque destabilization in apolipoprotein E-deficient mice. *Circulation* **115,** 2516–2525 (2007).
8. Bot, M. *et al.* Lysophosphatidic acid triggers mast cell-driven atherosclerotic plaque destabilization by increasing vascular inflammation. *J. Lipid Res.* **54,** 1265–74 (2013).
9. Saluja, R., Khan, M., Church, M. K. & Maurer, M. The role of IL-33 and mast cells in allergy and inflammation. *Clin. Transl. Allergy* **5,** 33 (2015).
10. Maruotti, N., Crivellato, E., Cantatore, F. P., Vacca, A. & Ribatti, D. Mast cells in rheumatoid arthritis. *Clin. Rheumatol.* **26,** 1–4 (2007).
11. Liu, J. *et al.* Genetic deficiency and pharmacological stabilization of mast cells reduce diet-induced obesity and diabetes in mice. *Nat. Med.* **15,** 940–945 (2009).
12. Tanaka, A., Nomura, Y., Matsuda, A., Ohmori, K. & Matsuda, H. Mast cells function as an alternative modulator of adipogenesis through 15-deoxy-delta-12, 14-prostaglandin J2. *Am. J. Physiol. Cell Physiol.* **301,** C1360-7 (2011).
13. Zhou, Y. *et al.* Leptin Deficiency Shifts Mast Cells toward Anti-Inflammatory Actions and Protects Mice from Obesity and Diabetes by Polarizing M2 Macrophages. *Cell Metab.* **22,** 1045–1058 (2015).
14. Finlin, B. S. *et al.* Mast Cells Promote Seasonal White Adipose Beiging in Humans. *Diabetes* **66,** 1237–1246 (2017).
15. Bartelt, A. & Heeren, J. Adipose tissue browning and metabolic health. *Nat. Rev. Endocrinol.* **10,** 24–36 (2014).
16. Laine, P. *et al.* Association Between Myocardial Infarction and the Mast Cells in the Adventitia of the Infarct-Related Coronary Artery. *Circulation* **99,** 361–369 (1999).
17. Kupreishvili, K. *et al.* Mast cells are increased in the media of coronary lesions in patients with myocardial infarction and may favor atherosclerotic plaque instability. *J. Cardiol.* **69,** 548–554 (2017).
18. Somasundaram, P. *et al.* Mast cell tryptase may modulate endothelial cell phenotype in healing myocardial infarcts. *J. Pathol.* **205,** 102–111 (2005).
19. Kwon, J. S. *et al.* The novel role of mast cells in the microenvironment of acute myocardial infarction. *J. Mol. Cell. Cardiol.* **50,** 814–825 (2011).
20. Ngkelo, A. *et al.* Mast cells regulate myofilament calcium sensitization and heart function after myocardial infarction. *J. Exp. Med.* **213,** 1353–1374 (2016).
21. Liao, C. *et al.* Cardiac mast cells cause atrial fibrillation through PDGF-A-mediated fibrosis in pressure-overloaded mouse hearts. *J. Clin. Invest.* **120,** 242–253 (2010).

22. De Jesus, N. M. *et al.* Atherosclerosis exacerbates arrhythmia following myocardial infarction: Role of myocardial inflammation. *Heart Rhythm* **12,** 169–178 (2015).

23. Rakusan, K., Sarkar, K., Turek, Z. & Wicker, P. Mast cells in the rat heart during normal growth and in cardiac hypertrophy. *Circ. Res.* **66,** 511–516 (1990).

24. Morrey, C. *et al.* Interaction between sensory C-fibers and cardiac mast cells in ischemia/reperfusion: activation of a local renin-angiotensin system culminating in severe arrhythmic dysfunction. *J. Pharmacol. Exp. Ther.* **335,** 76–84 (2010).

25. Koda, K. *et al.* Aldehyde dehydrogenase activation prevents reperfusion arrhythmias by inhibiting local renin release from cardiac mast cells. *Circulation* **122,** 771–781 (2010).

26. Hulsmans, M. *et al.* Macrophages Facilitate Electrical Conduction in the Heart. *Cell* **169,** 510–522.e20 (2017).

27. Yeong, P., Ning, Y., Xu, Y., Li, X. & Yin, L. Tryptase promotes human monocyte-derived macrophage foam cell formation by suppressing LXRalpha activation. *Biochim. Biophys. Acta* **1801,** 567–576 (2010).

28. Wang, J. *et al.* IgE actions on CD4+ T cells, mast cells, and macrophages participate in the pathogenesis of experimental abdominal aortic aneurysms. *EMBO Mol. Med.* **6,** 952–969 (2014).

29. Bot, M. *et al.* Lysophosphatidic acid triggers mast cell-driven atherosclerotic plaque destabilization by increasing vascular inflammation. *J. Lipid Res.* **54,** 1265–74 (2013).

30. Bot, M. *et al.* Atherosclerotic lesion progression changes lysophosphatidic acid homeostasis to favor its accumulation. *Am. J. Pathol.* **176,** 3073–3084 (2010).

31. Schober, A. & Siess, W. Lysophosphatidic acid in atherosclerotic diseases. *Br. J. Pharmacol.* **167,** 465–82 (2012).

32. Fukushima, N. & Chun, J. The LPA receptors. *Prostaglandins Other Lipid Mediat.* **64,** 21–32 (2001).

33. Yung, Y. C., Stoddard, N. C. & Chun, J. *LPA receptor signaling: pharmacology, physiology, and pathophysiology. Journal of lipid research* **55,** (2014).

34. Bagga, S. *et al.* Lysophosphatidic acid accelerates the development of human mast cells. *Blood* **104,** 4080–7 (2004).

35. Lundequist, A. & Boyce, J. A. LPA5 is abundantly expressed by human mast cells and important for lysophosphatidic acid induced MIP-1beta release. *PLoS One* **6,** e18192 (2011).

36. Foks, A. C., Lichtman, A. H. & Kuiper, J. Treating atherosclerosis with regulatory T cells. *Arterioscler. Thromb. Vasc. Biol.* **35,** 280–287 (2015).

37. Foks, A. C. *et al.* Differential effects of regulatory T cells on the initiation and regression of atherosclerosis. *Atherosclerosis* **218,** 53–60 (2011).

38. Tse, K., Tse, H., Sidney, J., Sette, A. & Ley, K. T cells in atherosclerosis. *Int. Immunol.* **25,** 615–622 (2013).

39. Jonasson, L., Holm, J., Skalli, O., Bondjers, G. & Hansson, G. K. Regional accumulations of T cells, macrophages, and smooth muscle cells in the human atherosclerotic plaque. *Arteriosclerosis* **6,** 131–138 (1986).

40. Gaudenzio, N., Laurent, C., Valitutti, S. & Espinosa, E. Human mast cells drive memory CD4+ T cells toward an inflammatory IL-22+ phenotype. *J. Allergy Clin. Immunol.* **131,** 1400–7.e11 (2013).

41. Sehra, S. *et al.* TH9 cells are required for tissue mast cell accumulation during allergic inflammation. *J. Allergy Clin. Immunol.* **136,** 433–40.e1 (2015).

42. Suurmond, J. *et al.* Communication between human mast cells and CD4(+) T cells through antigen-dependent interactions. *Eur. J. Immunol.* **43,** 1758–1768 (2013).

43. Kambayashi, T. & Laufer, T. M. Atypical MHC class II-expressing antigen-presenting cells:

can anything replace a dendritic cell? *Nat. Rev. Immunol.* **14,** 719–30 (2014).

44. Chai, O. H., Han, E.-H., Lee, H.-K. & Song, C. H. Mast cells play a key role in Th2 cytokine-dependent asthma model through production of adhesion molecules by liberation of TNF-alpha. *Exp. Mol. Med.* **43,** 35–43 (2011).
45. Tupin, E. *et al.* CD1d-dependent activation of NKT cells aggravates atherosclerosis. *J. Exp. Med.* **199,** 417–422 (2004).
46. van Puijvelde, G. H. M. & Kuiper, J. NKT cells in cardiovascular diseases. *Eur. J. Pharmacol.* (2017). doi:10.1016/j.ejphar.2017.03.052
47. Godfrey, D. I. & Kronenberg, M. Going both ways: immune regulation via CD1d-dependent NKT cells. *J. Clin. Invest.* **114,** 1379–1388 (2004).
48. Miyazaki, Y. *et al.* Effect of high fat diet on NKT cell function and NKT cell-mediated regulation of Th1 responses. *Scand. J. Immunol.* **67,** 230–237 (2008).
49. Huh, J. Y. *et al.* Deletion of CD1d in adipocytes aggravates adipose tissue inflammation and insulin resistance in obesity. *Diabetes* **66,** 835–847 (2017).
50. Teige, A. *et al.* CD1d-dependent NKT cells play a protective role in acute and chronic arthritis models by ameliorating antigen-specific Th1 responses. *J. Immunol.* **185,** 345–56 (2010).
51. Nakai, Y. *et al.* Natural killer T cells accelerate atherogenesis in mice. *Blood* **104,** 2051–2059 (2004).
52. Li, Y. *et al.* A CD1d-dependent lipid antagonist to NKT cells ameliorates atherosclerosis in ApoE-/- mice by reducing lesion necrosis and inflammation. *Cardiovasc. Res.* **109,** 305–317 (2016).
53. Kaartinen, M., Penttila, A. & Kovanen, P. T. Mast cells of two types differing in neutral protease composition in the human aortic intima. Demonstration of tryptase- and tryptase/chymase-containing mast cells in normal intimas, fatty streaks, and the shoulder region of atheromas. *Arterioscler. Thromb. a J. Vasc. Biol.* **14,** 966–972 (1994).
54. Kraft, S. *et al.* The tetraspanin CD63 is required for efficient IgE-mediated mast cell degranulation and anaphylaxis. *J. Immunol.* **191,** 2871–2878 (2013).
55. Burd, P. R. *et al.* Interleukin 3-dependent and -independent mast cells stimulated with IgE and antigen express multiple cytokines. *J. Exp. Med.* **170,** 245–257 (1989).
56. Guo, X. *et al.* Serum IgE levels are associated with coronary artery disease severity. *Atherosclerosis* **251,** 355–360 (2016).
57. Meng, Z. *et al.* Oxidized low-density lipoprotein induces inflammatory responses in cultured human mast cells via Toll-like receptor 4. *Cell. Physiol. Biochem.* **31,** 842–53 (2013).
58. Lagraauw, H. M. *et al.* Vascular neuropeptide Y contributes to atherosclerotic plaque progression and perivascular mast cell activation. *Atherosclerosis* **235,** 196–203 (2014).
59. Bot, I. *et al.* The neuropeptide substance P mediates adventitial mast cell activation and induces intraplaque hemorrhage in advanced atherosclerosis. *Circ. Res.* **106,** 89–92 (2010).
60. Wezel, A. *et al.* Mast cells mediate neutrophil recruitment during atherosclerotic plaque progression. *Atherosclerosis* **241,** 289–296 (2015).
61. Bot, I. *et al.* Mast cell chymase inhibition reduces atherosclerotic plaque progression and improves plaque stability in ApoE-/- mice. *Cardiovasc. Res.* **89,** 244–252 (2011).
62. Kondo, S. *et al.* Role of mast cell tryptase in renal interstitial fibrosis. *J. Am. Soc. Nephrol.* **12,** 1668–1676 (2001).
63. Nissen, S. E. Effect of intensive lipid lowering on progression of coronary atherosclerosis: evidence for an early benefit from the Reversal of Atherosclerosis with Aggressive Lipid Lowering (REVERSAL) trial. *Am. J. Cardiol.* **96,** 61F–68F (2005).

64. Nissen, S. E. *et al.* Effect of very high-intensity statin therapy on regression of coronary atherosclerosis: the ASTEROID trial. *JAMA* **295,** 1556–1565 (2006).

65. Whayne, T. F. J. PCSK9 inhibitors in the current management of atherosclerosis. *Arch. Cardiol. Mex.* **87,** 43–48 (2017).

66. Rayner, K. J. *et al.* Antagonism of miR-33 in mice promotes reverse cholesterol transport and regression of atherosclerosis. *J. Clin. Invest.* **121,** 2921–2931 (2011).

67. Foks, A. C. *et al.* Interruption of the OX40 – OX40 Ligand Pathway in LDL Receptor – Deficient Mice Causes Regression of Atherosclerosis. (2013). doi:10.4049/jimmunol.1200708

68. Kita, T. *et al.* Regression of atherosclerosis with anti-CD3 antibody via augmenting a regulatory T-cell response in mice. *Cardiovasc. Res.* **102,** 107–117 (2014).

69. Ridker, P. M., Thuren, T., Zalewski, A. & Libby, P. Interleukin-1beta inhibition and the prevention of recurrent cardiovascular events: rationale and design of the Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS). *Am. Heart J.* **162,** 597–605 (2011).

70. Ridker, P. M. *et al.* Antiinflammatory Therapy with Canakinumab for Atherosclerotic Disease. *N. Engl. J. Med.* (2017). doi:10.1056/NEJMoa1707914

Curriculum Vitae

Eva Kritikou was born in Athens, Greece, on the 28th of October, 1984. She spent her early childhood between Athens and Naxos island. In 2002, she graduated from the American College of Greece and moved to Patras where she obtained a Bachelor's Degree in Biology from the University of Patras, in 2010. She performed her bachelor's research project in the Medical School and the department of Physiology, under Dr. S. Taraviras. In her project she investigated the proliferation and apoptosis mechanisms of neural progenitor cells. Her work was included in a research paper that appeared in the journal *Stem Cells*.

In early 2011, upon completion of her bachelor studies she travelled to Leiden to follow a Master's research program in Biomedical Sciences, at the Leiden University Medical Center. There she got intrigued by immunological research, which became her focus thereafter. Her first Master's project in the department of Molecular Cell Biology, was supervised by Prof. Dr. M.J. Goumans and involved the altering of differential macrophage subsets. Her Master's thesis project, performed in the department of Pathology under Dr. A. Gorter and Dr. E. Jordanova, examined the role of IL-17^{+} cells in human cervical cancer. Part of that work was presented in a publication on the journal *Oncoimmunology*.

During her PhD studies, she aimed to explore the challenging role of the immune system in atherosclerosis. She performed her doctorate in the division of Biopharmaceutics, LACDR, Leiden University, under Prof. Dr. J. Kuiper and Dr. I. Bot. Thereupon, she investigated the role of mast cells in atherosclerosis, which was reviewed in her publication on the *European Journal of Pharmacology*. In addition, she researched the lipid modifications on the immune system and this part of her studies was published in the journal *Scientific Reports.* For her work, she received a travel award to participate in the 85th European Atherosclerosis Society Congress. Moreover, she was awarded a poster prize at the LACDR Spring Symposium for her project on mast cells as antigen presenting cells in atherosclerosis and, she was invited to present her work at the Gordon Atherosclerosis Seminar of 2017.

Upon obtaining her doctorate degree, Eva will progress as a post-doctoral research fellow in the laboratory of Dr. A.H. Lichtman at the Brigham and Women's Hospital, Harvard Medical School, Boston, MA, United States of America.

Publications

Papers & abstracts:

Kritikou E, van der Heijden T, Swart M, van Duijn J, Slütter B, Smeets HJ, Maffia P, Kuiper J, Bot I. *Hypercholesterolemia induces a direct interaction between mast cells and CD4⁺ T-cells in atherosclerosis. Atherosclerosis* 2017; 263:e124 (published abstract)

Kritikou E, van Puijvelde GHM, van der Heijden T, van Santbrink PJ, Swart M, Schaftenaar FH, Kröner MJ, Kuiper J, Bot I. *Inhibition of lysophosphatidic acid receptors 1 and 3 attenuates atherosclerosis development in LDL-receptor deficient mice. Sci Rep.* 2016; 6:37585

Kritikou E, Kuiper J, Kovanen PT, Bot I. *The impact of mast cells on cardiovascular diseases. Eur J Pharmacol.* 2016; 778:103-15

Kritikou E, van der Heijden T, Swart M, van Duijn J, Slütter B, Wezel A, Smeets HJ, Maffia P, Kuiper J, Bot I. *Hypercholesterolemia induces a direct interaction between mast cells and CD4⁺ T-cells in atherosclerosis.* (manuscript submitted for publication)

Kritikou E, van Duijn J, Nahon J, van der Heijden T, Bouwman M, Groeneveldt C, Schaftenaar FH, Kröner MJ, Kuiper J, van Puijvelde GHM, Bot I. *Disruption of a CD1d-mediated interaction between mast cells and NKT cells aggravates atherosclerosis.* (manuscript submitted for publication)

Kritikou E, Schaftenaar FH, van der Heijden T, van Puijvelde GH, Nahon J, van Duijn, Kröner MJ, Yagita H, Kuiper J, Foks A, Bot I. *The role of CD48 in atherosclerosis.* (manuscript in preparation)

Kritikou E, van der Heijden T, van Puijvelde GHM, Swart M, Kröner MJ, Kuiper J, Bot I. *Mast cell depletion in advanced atherosclerosis does not induce plaque regression* (manuscript in preparation)

Kritikou E, Slütter B, van Duijn J, Foks A, van der Heijden T, Wezel A, Smeets HJ, Kuiper J, Bot I. *Phenotypic characterization of human intraplaque mast cells using flow cytometry* (manuscript in preparation)

van Duijn J, Kritikou E, Benne N, van der Heijden T, van de Puijvelde GHM, Kröner MJ, Schaftenaar FH, Foks A, Wezel A, Smeets HJ, Yagita H, Bot I, Jiskoot W, Kuiper J, Slütter B. *CD8⁺ T-cells contribute to lesion stabilization in advanced atherosclerosis by limiting macrophage content and CD4⁺ T-cell responses* (manuscript submitted for publication)

van der Heijden T, Kritikou E, Venema W, van Duijn J, van Santbrink PJ, Slütter B, Foks A, Bot I, Kuiper J. *NLRP3 inflammasome inhibition by MCC950 reduces atherosclerotic lesion development in apoE deficient mice. Arterioscler. Thromb. Vasc. Biol.* 2017; 37(8):1457-1461.

Punt S, Fleuren GJ, Kritikou E, Lubberts E, Trimbos JB, Jordanova ES, Gorter A. *Angels and demons: Th17 cells represent a beneficial response, while neutrophil IL-17 is associated with poor prognosis in squamous cervical cancer. Oncoimmunology,* 2015; 4(1):e9845349

Punt S, Houwing JJ, Schulkens IA, Thijssen VL, Osse EM, Kritikou E, de Kroon CD, Griffioen AW, Fleuren GJ, Gorter A, Jordanova E. *A high IL6/IL17 ratio combined with low IL5 expression is correlated with poor survival in squamous cervical cancer. Journal for ImmunoTherapy of Cancer* 2014; 2(Suppl 3):P232 (published abstract)

Spella M, Kyrousi C, Kritikou E, Stathopoulou A, Guillemot F, Kioussis D, Pachnis V, Lygerou Z, and Taraviras S. *Geminin regulates cortical progenitor proliferation and differentiation. Stem Cells,* 2011; (8):1269-82

(Inter)national Presentations:

2017: BPS Master's symposium – LACDR, Leiden, The Netherlands
(*oral presentation)*

Gordon Research Conference-Atherosclerosis, Sunday River, ME, USA
(poster presentation)

Gordon Research Seminar –Atherosclerosis
(*oral presentation*)

LACDR Spring Symposium, Leiden, The Netherlands
(*poster presentation & prize winner)*

European Atherosclerosis Society congress, Prague, Czech Republic
(travel grant award & poster presentation)

2016: IVBM-NAVBO meeting, Boston, MA, USA
(poster presentation)

2015: NVVI-Dutch Society for Immunology, Noordwijkerhout, The Netherlands
(*poster presentation)*

6th Rembrandt Symposium, Noordwijkerhout, The Netherlands
(*poster presentation)*

Leiden Vascular Medicine meeting, Leiden, The Netherlands
(oral presentation)

2014: Scandinavian Society for Atherosclerosis, Humlebaek, Denmark
(poster presentation)

Leiden Vascular Medicine meeting, Leiden, The Netherlands
(oral presentation)

5th Rembrandt Symposium, Noordwijkerhout, The Netherlands
(*poster presentation)*

Courses and training:

Advanced Immunology (LIFI) course
Dutch Society for Immunology annual symposium - Lunteren
Atherothrombosis and Coagulation
Clinical Research in practice
Animal Handling course (Article 9-EU regulations)
Bioinformatics
Introductory Course on Drug Research
How to write a research proposal
Scientific Conduct
Communication in Science
Data management
Introduction to teaching and supervision
Press Release course
Time management