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Mast cells as immune regulators in atherosclerosis

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Chapter 1

General introduction - Atherosclerosis

A brief history of atherosclerosis

In the course of human history, infectious diseases, spread by pathogenic microorganisms, are held accountable for the loss of millions worldwide¹. The rise of the 20th century however, found humankind in the brink of medical breakthrough with vaccine development² 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 disease⁴.

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 2030⁵. 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; atherosclerosis⁷. Atherosclerotic disease has been described in humans since the ancient years⁸, but it is the modern-era nutritional habits⁹ that deem it the prevailing component of human mortality¹⁰.

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 aorta¹¹. 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 diet¹². 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, appear¹⁸. The pathology of atherosclerosis is established with the formation of multiple lipid-rich plaques in the large branches of the arterial tree¹⁹. The most susceptible sites within the human body are the ones most sensitive to shear stress changes²⁰ 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 compartments²¹. 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-cadherin²⁶ and upregulation of adhesion molecules such as VCAM-1 or ICAM-1²⁷. The damage inflicted upon the endothelial cell junctions permits the lipoprotein molecules to infiltrate the subendothelial space²⁸, bind proteoglycan molecules^{29,30} and undergo chemical modifications³¹. This process retains the lipoprotein molecules in the subendothelial space. Concurrently, loss of the vascular EC pattern³² and adhesion molecule upregulation³³ but also chemokine secretion upon EC activation³⁴, initiates the permeation of inflammatory immune cells in the area¹⁷. Accumulated phagocytes are able to digest the modified LDL molecules giving rise to a thin intimal layer called fatty streak³⁵. Fatty streaks can be detected already at an infant age, usually depending on the genetic and environmental background of the mother³⁶. These early atheromata are harmless and can disappear during development, for instance by lowering serum cholesterol levels³⁷. 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 stages³⁸.

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 place⁴¹. However, as more and more lipids accumulate in the area, this programmed apoptotic mechanism begins to fail, while being replaced by non-specific secondary necrosis⁴². Necrotic material accumulation and destruction of the cellular plaque composition leaves behind debris, termed necrotic core regions^{43,44}, and increased plaque burden⁴⁵. Upon further progression, the atherosclerotic plaque is also characterized by an increase in smooth muscle cell content⁴⁶, extracellular matrix protein synthesis and degradation⁴⁷ and neovessel formation⁴⁸. 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 outskirts 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 plaque⁵³, increases the inflammatory burden in the area, by giving passage to immune cell infiltration inside the intima⁵⁴. Furthermore, the plaque site is subjected to a high risk for microvessel leakage which can lead to intraplaque hemorrhage⁵⁵. Advanced plaques do not have the ability to recede³⁷, 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 episode⁶⁰ (**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 stroke⁶¹. 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 plaque⁶², as well as balloon angioplasty, with or without stent placement^{63,64}, to restore the normal blood flow; alternatively, vascular bypass operation⁶⁵ helps to divert the blood circulation *via* a non-occluded vessel. Yet, all these intervention methods usually lead to complications, like restenosis⁶⁶. 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 regression⁶⁹.

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 stratification⁷⁰ 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 rupture⁷¹.

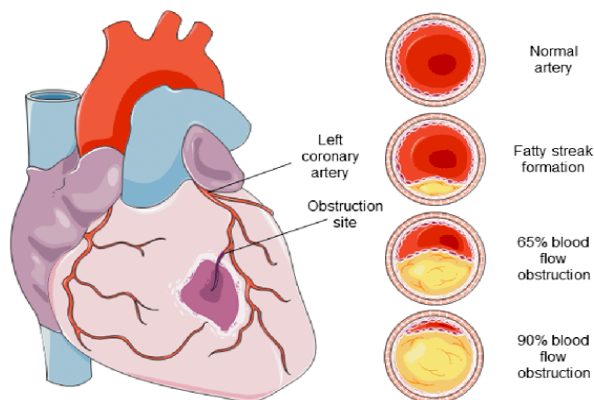


Figure 1: Myocardial infarction site. Coronary arteries show high preponderance in atherosclerotic plaque formation. Fatty streaks are harmless intima protrusions with reversible plaque formation. However, increasing lipid deposition leads to advanced plaques, or fibroatheromas, characterized by necrotic 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 choices⁹, also depends on various determinants acting independently or simultaneously, such as diabetes⁷², hypertension⁷³, smoking⁷⁴ or even an individual's gender⁷⁵. 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 models⁷⁶. Rabbits⁷⁷ or non-human primates⁷⁸ 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 hypercholesterolemia⁷⁹; 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 studies⁸⁰. 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 thrombi⁸¹; 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 production⁸², bile acid synthesis⁸³ and cell membrane structure⁸⁴. Plasma cholesterol levels are influenced, up to an extent, by the cholesterol amount absorbed through the diet⁸⁵. Dietary cholesterol absorption is mediated in part by the intestines, the function of which has gained increasing attention in the field of atherosclerosis⁸⁶. However, cholesterol homeostasis is mainly regulated by the liver⁸⁷, a key organ responsible for its synthesis and degradation⁸⁸. In brief, the synthesis of cholesterol is controlled by SREBP-proteins⁸⁹ and the enzyme HMG-CoA reductase⁹⁰. Cholesterol molecules are delivered to the blood in the form of VLDL or its hydrolyzed product, LDL⁹¹ (**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 excretion⁹³.

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 cell⁹⁴. For example, LXR can inhibit the uptake of cholesterol by negatively regulating the expression of LDLr⁹⁵, can interrupt cholesterol synthesis by acting on the SREBPs⁹⁶, but can also increase the transcription of the cholesterol efflux proteins ABCA1⁹⁷ and ABCG1⁹⁸ which release the accumulated intracellular cholesterol. The action of LXR on ABCA1⁹⁹ and ABCG1¹⁰⁰ 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 HDL¹⁰¹.

The function of HDL is considered mainly beneficial¹⁰², 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 regression¹⁰⁵⁻¹⁰⁷. 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 progression¹¹².

Specifically, lipoprotein molecules that have already penetrated the endothelial cell barrier, and have been further subjected to modifications such as oxidation³¹, are subsequently taken up by locally proliferating macrophages, which then become foam cells¹¹³. Intraplaque macrophages internalize oxidized LDL through receptors such as protein CD36, that serves as a scavenger receptor¹¹⁴. 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 toxicity¹¹⁵. Cholesterol esterification is followed by circles of hydrolysis which prepare the molecules for efflux from the cell¹¹⁶. 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 particles¹¹⁷, in a process named reverse cholesterol transport^{118,119}. These particles can then be internalized by the scavenger receptor SR-BI in the liver¹²⁰ and subsequently get cleared away by the intestines¹²¹. 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 apoptosis¹²². As explained before,

incessant local apoptosis intensifies the already initiated inflammatory response in the arterial wall leading thus to the formation of advanced atherosclerotic plaques⁴¹.

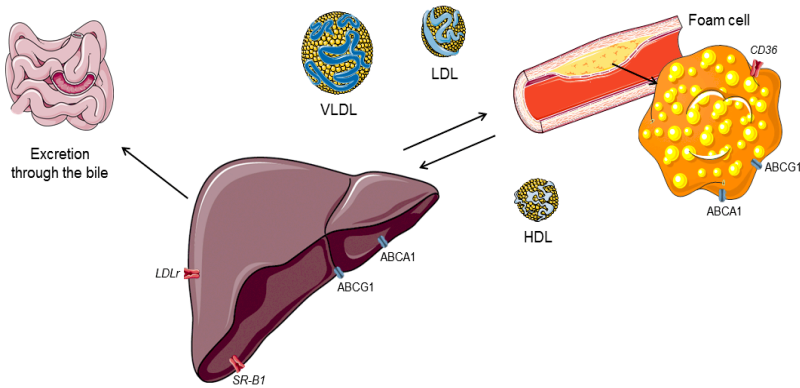


Figure 2: Cholesterol metabolism pathway. Cholesterol is synthesized in the liver and released in the circulation *via* (V)LDL molecules. High concentration of serum cholesterol leads to the accumulation of low-density lipoprotein particles in the subendothelial space where they get modified and internalized 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-B1* 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 cassette A1/G1, SR-B1: 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*^{-/-}) mice⁷⁶. 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 formation¹²³. 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 liver¹²⁴. 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 pathways¹²⁵. It is important to keep this in mind¹²⁶, 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 disease¹²⁷.

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 stage¹²⁸. The immune system response generally, as well as in atherosclerosis, is classified into a fast reaction elicited by innate immune cells¹²⁹, such as neutrophils, monocytes and mast cells, and a slower, more specific, reaction through adaptive immune T and B lymphocytes and NKT cells¹³⁰ (**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 it¹³¹.

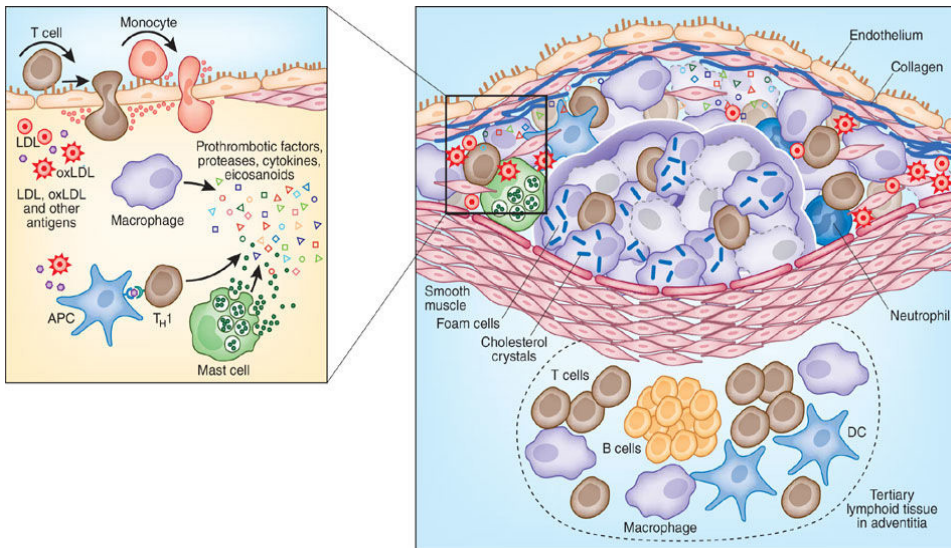


Figure 3: Immune cell types involved in atherosclerosis. The main cellular constituents of an atherosclerotic plaque are the macrophages. Macrophages proliferate locally in the plaque after their monocytic precursors penetrate the endothelial wall layer. Macrophages are able to internalize oxidized LDL, 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 damage¹³⁴. 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 damage¹³⁵. In the case of atherosclerosis this happens as soon as lipoproteins accumulate in the subendothelial space¹³⁶. 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 signals¹³⁷. MCP-1, as the name suggests, attracts monocytes in the plaque area, guided there by their expression of chemokine receptor CCR2¹³⁸. 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 membrane¹³⁴. In humans, monocytes are sorted into three classes based on the expression levels of proteins CD16 and CD14¹⁴¹ with one of the subsets considered a predictor for future coronary events¹⁴². In mice, this classification relies on the expression of protein Ly6C¹⁴³. 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 development¹⁴⁶ upon migration in the inflamed tissue. For example, Ly6C^{high} monocyte migration regulated by protein RP105¹⁴⁷ was shown to positively affect atherogenesis¹⁴⁸.

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 appearance¹⁵². 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 damage¹⁵³. 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 cells¹⁵⁵ and initiate a downstream cascade that

leads to the secretion of cytokines like IL-1 β and IL-6, which further augment the inflammation in the tissue by acting on the T cells¹⁵⁶. In addition, macrophages are a rich source of matrix metalloproteinases¹⁵⁶, which degrade the extracellular matrix of the plaque and affect plaque stability¹⁵⁷. The phagocytic capacity of atherogenic proteins by macrophages, is proven to induce endoplasmic reticulum stress and thereby leads to cell apoptosis¹⁵⁸. As mentioned before, induction of apoptosis during initial atherosclerosis stages can be beneficial, while persistent apoptosis at a later stage increases plaque development¹⁵⁹ and instability¹⁶⁰.

Macrophages appear in multiple subsets during atherosclerosis development, defined by their differential responses¹⁶¹. In the past, it was thought that macrophages are classified as either classical, inflammatory, M1 macrophages or alternative, healing, M2 macrophages¹⁶². However, more and more studies indicate that there are in fact multiple macrophage subsets and each one acts differently within the atherosclerotic plaque¹⁶³. M1 macrophages were abundantly found in the plaques of patients suffering an ischemic episode, as compared to M2 macrophages which were increased in stable plaques¹⁶⁴. In general, M1 macrophages are reported to enhance inflammation within the plaque, for instance through the production of toxic reactive oxygen species¹⁶⁵. On the other hand, M2 macrophages have recently been proven responsible for atherosclerotic plaque regression¹⁶⁶. 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 proatherogenic¹⁶⁷. 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 properties¹⁶⁸, 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 CD11c¹⁷⁰ and F4/80¹⁷¹ 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 phenotype¹⁷⁶. 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 pathology¹⁷⁷; 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 atheroprotective¹⁷⁸. On the contrary, pDCs seem to affect atherosclerosis progression in a positive manner¹⁷⁹. Importantly, while uptake of oxLDL by DCs is known to elicit a proinflammatory DC-action¹⁸⁰, 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 development¹⁸¹. In addition, oxLDL-induced apoptosis on DCs has also been found to be atheroprotective¹⁸², 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 arteries¹⁸³, while it was upregulated during atherosclerosis regression¹⁸⁴. 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 plaques¹⁸⁵.

Dendritic cells normally populate lymphoid tissues¹⁸⁶ and are classified as professional antigen presenting cells (APCs)¹⁸⁷. As such, in atherosclerosis¹⁸⁸, 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 place¹⁹¹. Signal I is provided through specific antigen presentation by the MHC-machinery to the T cell receptor¹⁹². While MHC-I is expressed in all cells, since it is conserved for the fight against viral responses¹⁹³, MHC-II is expressed mainly by APCs¹⁸⁷, but also, upon induction, by some non-conventional APCs, like mast cells¹⁸⁷. 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 respectively¹⁹⁴. The fashion of the costimulatory interplay is crucial since it can alter the T cell response from an effector into an inhibitory one¹⁹⁵. 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 molecules¹⁹⁶, usually derived from activated APCs, for example in the form of IL-12, TNF α , 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 subsets¹⁹⁹.

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 times²⁰⁰ 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 mature and present antigens to naïve lymphocytes in the area²⁰¹. 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 activation²⁰². In contrast, apoB100, a particle carried by (V)LDL molecules, has been found to induce a protective adaptive immune response through T cells²⁰³, similarly to heat shock proteins 60/65²⁰⁴. This is particularly interesting since it means that specific tolerogenic peptides can be the basis for vaccination strategies against atherosclerosis²⁰⁵. In fact, very recently three new peptides of the apoB molecules were discovered to confer protection against the development of atherosclerosis²⁰⁶, 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 spleen²⁰⁷. 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 locally²¹⁴.

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 cells²¹⁵, while interaction through MHC-II activates CD4⁺T cells²¹⁶. This is usually dependent on the means through which APCs have acquired the antigen and the length of the peptide²¹⁷.

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 numbers²¹⁸. CD8⁺ T cells are mainly suggested to act in a cytotoxic manner, through the secretion of cytokines like interferon- γ (IFN γ)²¹⁹ and granzyme B²²⁰ 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 protectively²²¹. 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 properties²²². 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-10²²³. 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 receive²²⁴. 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-4²²⁵. 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 plaque²¹⁴ and they are proatherogenic²²⁶, mainly through the secretion of cytokine IFN γ 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-4²²⁸ reported to counteract the actions of IFN γ ²²⁹. Even though T_{H2} cells have been proven to neutralize the effect of T_{H1} cells²²⁶, 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 formation²³⁰ and act in a proatherogenic manner²³¹. On the contrary, IL-5 seems to exert a protective effect, by positively affecting the production of athero-protective antibodies by B cells²³². Another important CD4⁺ T cell subset is the T_{H17}. These cells arise upon the influence of cytokines IL-6, IL-1 β , TGF β and IL-21, and through activation of transcription factor ROR γ t, and are considered the main secretors of cytokine IL-17²³³. 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-17²³⁴, a follow-up report did not find any effect of IL-17 in atherosclerotic plaque development²³⁵. Interestingly, IFN γ secretion by T_{H17} cells seems to associate with the progression of coronary atherosclerosis²³⁶, with HDL-particles reported to attenuate T_{H1} and T_{H17} responses²³⁷. 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 type²³⁸. The role of T_{REG} cells is essential in atherosclerosis, since they are the main athero-protective cell type²³⁹. 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-10²⁴⁰. The presence of T_{REG} cells is reduced upon atherosclerosis progression²⁴¹ and they are negatively associated with the development of myocardial infarction²⁴². Their anti-atherogenic manner is attributed mainly to the secretion of IL-10 which can skew macrophages into an M2-phenotype²⁴³ and hamper T_{H1}²⁴⁴ responses. In fact, T_{REG} cells inhibit inflammatory responses upon atherosclerosis progression while stabilizing plaques during atherosclerosis regression²⁴⁵. Thus, T_{REG} cells have become a very attractive target for immune-cell based therapies in atherosclerosis²⁴⁶.

Finally, there are accumulating reports on additional T cell subsets and their role in cardiovascular syndromes, such as T_{H22} and T_{H9} cells²⁴⁷. 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 $\gamma\delta$ T cells, mast cells and neutrophils²⁴⁸. 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 cells²⁴⁹.

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 subset²⁵⁰. For example, GATA-3 expression on T_{H1} cells can effectively alter them into a T_{H2} phenotype²⁵¹. More specifically for atherosclerosis, it was recently reported that inside progressing plaques, T_{REG} cells acquire a dysfunctional IFN γ secreting phenotype²⁵². 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) cells²⁵³. The NKT cell population migrates from the thymus into the periphery, particularly the spleen and liver, and is reactive to endogenous and exogenous lipid antigens²⁵⁴. NKT cells possess a special T cell receptor chain²⁵⁵ 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 development²⁵⁸; particularly during the initial phase²⁵⁹, when the adaptive response has not yet been established. NKT cells are considered to be proatherogenic²⁶⁰ due to their potent cytokine secretion, that positively enhances plaque inflammation, particularly through $IFN\gamma$ ²⁶¹ and granzyme B²⁶² secretion. However, they have been found to act also at later stages²⁶³, 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 formation²⁶⁴, has been found to affect CD1d expression²⁶⁵, suggesting that there is an endogenous lipid ligand present in atherosclerosis that activates NKT cells²⁶⁶. It is important to note that different ligands can affect NKT cells in a way that can be either proinflammatory or anti-inflammatory²⁶⁰. 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 atherosclerosis²⁶⁷. B cells are the antibody producing machinery of the body and in atherosclerosis they have been found to produce both proatherogenic IgG2c²⁶⁸, as well as, anti-atherogenic IgM²⁶⁹ antibody fragments. The overall role of B cells in atherosclerosis was initially thought to be protective, since their absence increased plaque development²⁷⁰. However, these cells are also distinguished in various subsets with contrasting abilities²⁷¹. Mainly, B cells are separated into B1 cells, which are considered atheroprotective^{269,272}, and B2 proatherogenic cells²⁷³. In atherosclerosis, different B cell antibody fragments show different effects. For example, IgM has been found to be indispensable in the protection against atherosclerosis²⁷⁴, while apoB100-specific IgG fragments are

proatherogenic²⁷⁵. 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 activation²⁷⁶. In addition, the GM-CSF producing IRA B cells have also been reported to be proatherogenic²⁶⁸. On the contrary, the contribution of IL-10 secreting B cells in atherosclerosis is still somewhat unclear²⁷⁷ with one study postulating an atheroprotective role²⁷⁸, while another did not report an effect in atherosclerosis development²⁷⁹. B cells reside in large amounts in the spleen and lymph nodes, particularly in the follicular centers and marginal zone²⁸⁰, but in atherosclerosis they can also be found inside the peritoneal cavity²⁸¹, which is rich also in mast cells²⁸² 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 neutrophils²⁸³. 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 receptors²⁸⁴. Neutrophils are characterized by their very short lifespan²⁸⁵ and are rich in chemokine receptors, the most known being CXCR2, and CXCR4²⁸⁶. However, under chronic inflammatory conditions, neutrophils can upregulate additional chemokine receptors, such as CCR2 and CXCR3²⁸⁷. In atherosclerosis they increase upon hyperlipidemia and accelerate the early phase of plaque development²⁸⁸. Neutrophils can crawl on the endothelial cell wall of arterial sites exposed to high shear stress²⁸⁹, infiltrate the subendothelium^{290,291} and inflict local damage, increasing therefore the penetration rate of additional immune cells, like monocytes²⁹². They are considered proatherogenic due to their release of proteases and production of MPO²⁹³ and ROS²⁹⁴, 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 metalloproteinases²⁹⁵. In support of their role during end-stage atherosclerosis, neutrophils are positively associated with acute coronary events²⁹⁶. 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 damaging²⁹⁷. 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 proatherogenic³⁰¹. 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 interaction³⁰².

3.7 Mast cells

One of the most important granulocytic populations involved in atherosclerosis are the mast cells³⁰³. Mast cells are present within the arterial wall at low numbers, even under normal conditions, but accumulate in the area upon atherosclerosis progression³⁰⁴. 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 CD117³⁰⁷. Furthermore, mast cells are recognized by their distinct granular mediators, neutral proteases tryptase and chymase, histamine as well as proinflammatory cytokines like IL-6, TNF α and IFN γ ³⁰⁸. In the circulation mast cells can be found solely as progenitors and, similar to macrophages, their end-stage maturation takes place only within tissues³⁰⁹. 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 chymase³¹⁰. The activation of mast cells can occur *via* multiple ways. The typical pathway involves binding of an antigen sensitized-IgE antibody onto their characteristic Fc ϵ -receptor³¹¹. However, mast cells have been reported to get activated *via* additional pathways, such as for instance through TLRs³¹², complement receptors³¹³ and neuropeptide receptors³¹⁴; all activation pathways that have been also implicated in atherosclerosis.

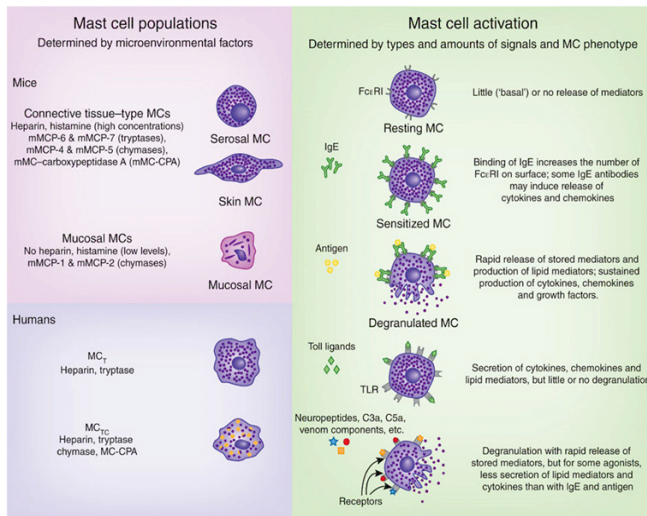


Figure 4. Mast cell subsets and activation pathways. Mast cells are distinguished in various subtypes depending on their place of residence and microenvironment. Mast cell activation can occur through the classical pathway involving binding of an IgE fragment onto their characteristic Fc ϵ R, but can also occur through 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 destabilization³⁰⁶, through their protease secretion³¹⁵ which can degrade the extracellular matrix³¹⁶. They are also reported to enhance foam cell formation, by acting on the cholesterol efflux mechanism³¹⁷. Mast cells possess many chemokine receptors, such as CCR2³¹⁸, 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 apoB100³¹⁹.

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 eosinophils³²⁰, NK cells³²¹, innate lymphoid cells³²², MDSCs³²³, or $\gamma\delta$ 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 disease³²⁵. 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-modification³²⁶. LPA has been proven to activate an array of immune cells such as CD4⁺ T cells³²⁷ and mast cells³²⁸. In addition, it was only recently reported to enhance foam cell formation, for example through inhibiting SR-BI³²⁹, 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.

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