Lipids, inflammation and atherosclerosis

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Chapter 1

GENERAL INTRODUCTION

LIPIDS

The accumulation of lipids, especially cholesterol, in the vessel wall is a hallmark of atherosclerosis. Cholesterol is a component of cell membranes and is essential for membrane fluidity and synthesis of steroid hormones, vitamins and bile acids. Homeostasis of cholesterol is maintained by *de novo* synthesis, by absorption from diet, by catabolism into bile acids and other steroids and by excretion into bile. Cholesterol and other lipids, such as triglycerides (TG) and phospholipids (PL) are incorporated in lipoproteins for transportation in the blood circulation. Lipoproteins are an assembly of proteins and lipids, consisting of a hydrophobic core of cholesteryl esters (CE) and TG surrounded by a hydrophilic monolayer of PL, free cholesterol and apolipoproteins (apo)[1]. The major lipoprotein classes include chylomicrons, very low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL)[1].

Dietary lipids are emulsified and hydrolyzed in the lumen of the intestine, absorbed by enterocytes and assembled into triglyceride-rich chylomicrons containing apoB48[2,3]. In the blood circulation, the chylomicrons acquire apoCs and apoE from HDL and triglycerides within the chylomicron core are lipolyzed by lipoproteinlipase (LPL) leading to the release of (free) fatty acids, which are taken up by surrounding tissue[2,3]. The resulting CE-rich chylomicron remnants are eliminated from the circulation by the liver, mainly via the LDL receptor (LDLr) and LDLr related protein (LRP)-1[4].

In the liver, cholesterol and triglycerides from internalized chylomicron remnants can be converted to bile acids or, together with newly synthesized lipids, used for the assembly of TG-rich VLDL containing apoB100[5]. VLDL acquires apoCs and apoE and in the blood circulation, triglycerides in the core of VLDL are subject to lipolysis by LPL, resulting in the formation of the more CE-rich IDL and free fatty acids, which are provided to the surrounding tissue[6]. IDL is either removed by the liver via LDLr and LRP-1 or is processed further to mature LDL containing apoB100 as sole apolipoprotein. The LDLr plays a major role in LDL mediated cholesterol delivery to peripheral tissue and clearance of LDL from the circulation via the liver[7], but, upon modification, LDL can also be taken up via scavenger receptors[8].

HDL is produced primarily by the liver and intestines and starts with the secretion of lipid-poor apoA-I, which acquires PL and unesterified cholesterol resulting in the formation of nascent discoidal pre-β HDL[9]. Free cholesterol from peripheral cells, especially macrophages, is efficiently taken up by nascent HDL, after which it is converted to CE by lecithin:cholesteryl acyltransferase (LCAT), leading to the formation of

spherical HDL[10]. The cellular efflux of cholesterol is mediated by ATP-binding cassette transporters (ABC), of which ABCA1 and ABCG1 are the key players in this process[11,12]. Mature HDL delivers cholesterol to the liver via scavenger receptor class B, type I (SR-BI) without internalization of the HDL particle itself, but via selective CE uptake[13]. In humans and rabbits, but not in mice, cholesteryl ester transfer protein (CETP) creates a major diversion from this route. In exchange for TG, CETP facilitates transfer of CE from HDL to apoB containing particles, which in turn can be taken up by the liver[14]. Ultimately, excess liver cholesterol can be excreted as neutral sterols and bile acids into bile for removal via the feces. The efflux of peripheral cholesterol to HDL, and its transportation to the liver for excretion from the body, is called reverse cholesterol transport[15]. A schematic overview of lipoprotein metabolism is given in **Fig. 1**.

Serum cholesterol levels are largely influenced by synthesis and secretion of VLDL and HDL and by removal of cholesterol from the body via bile[5,16]. These processes occur in the liver, which therefore is the key organ in serum lipid homeostasis. The liver consists mainly of three cell types: parenchymal cells, endothelial cells and Kupffer cells[17]. Of these cell types, parenchymal cells occupy almost 80% of the total liver volume and perform the majority of the numerous liver functions, including cholesterol uptake and metabolism for biliary excretion and VLDL and HDL synthesis and secretion. The transcription of a variety of genes involved in cholesterol homeostasis, such as lipoprotein receptors, enzymes in cholesterol biosynthesis and cholesterol transporters, is regulated by several transcription factors and nuclear receptors including peroxisome proliferator-activated receptors (PPAR), liver X receptors (LXR) and sterol regulatory element binding proteins (SREBP)[18-20].

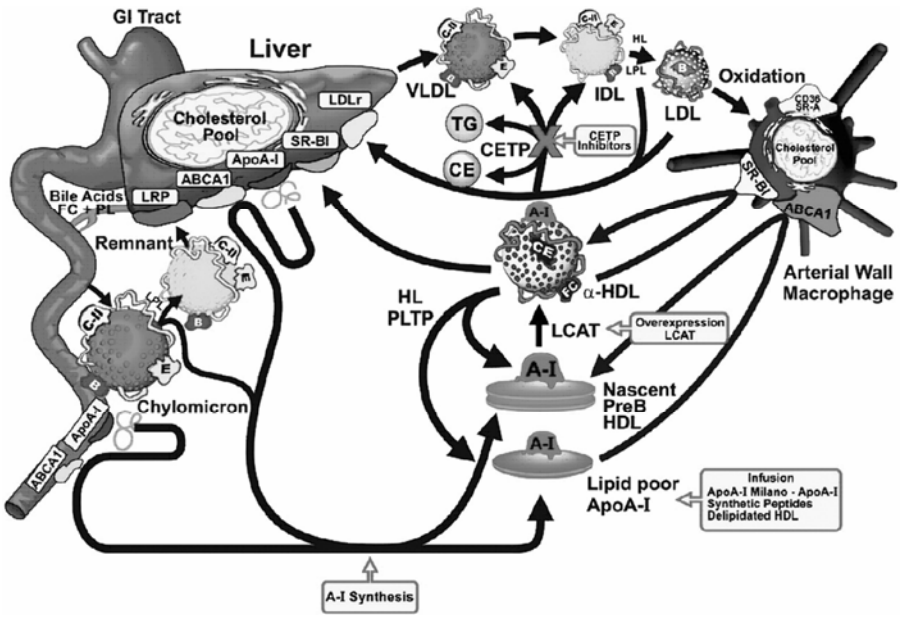

Fig. 1: Schematic overview of lipoprotein metabolism (Adapted from Brewer[21])

INFLAMMATION

Immune system

Although atherosclerosis has traditionally been regarded to simply reflect the deposition of lipids within the vascular wall of medium sized to large arteries, it is now widely accepted that immune responses participate in and can regulate atherosclerotic lesion development[22-24]. The immune system (**Fig. 2**) is one of the major systems in the body composed of many interactive, specialized cell types that collectively protect the body from bacterial, parasitic, fungal, viral infections and from the growth of tumor cells. During development, the immune system has learned to discriminate between self and non-self, resulting in self tolerance, which prevents the body from mounting an immune attack against its own tissues. However, the immune system is involved in the clearance of apoptotic cells from the body and, in case of autoimmune diseases, it can be activated by endogenous stimuli[25]. Inflammation is one of the first responses of the immune system to infection involving the recruitment of immune cells to the site of injury. In addition, an inflammatory reaction serves to establish a physical barrier against the spread of infection and to promote healing of any damaged tissue following the clearance of pathogens. If a pathogen overcomes the exterior defenses (such as skin) and invades the body, it first encounters cells of the innate immune system, which detect and often eliminate the invader before it is able to reproduce and cause potentially serious injury to the host. Innate immunity involves several different cell types, most importantly those of the mononuclear phagocyte lineage, such as macrophages. Macrophages express receptors that recognize a broad range of molecular patterns foreign to the mammalian organism but commonly found on pathogens. These pattern-recognition receptors include various scavenger receptors and Toll-like receptors (TLR)[26,27]. Other cell types of the innate immune system include NK cells, mast cells, neutrophils and dendritic cells.

If a pathogen is able to successfully evade the innate immune cells, the immune system activates an adaptive immune response conducted by T and B lymphocytes. Adaptive immunity recognizes specific molecular structures (antigens) presented via major histocompatibility complex (MHC) classes II by antigen presenting cells of which macrophages are the major ones in atherosclerosis. Once T cells recognize an antigen presented to them, they initiate adaptive immune responses against this specific antigen[28]. These responses include direct killing of antigen bearing cells by cytotoxic T lymphocytes, stimulation of B cells to produce antibodies against the antigen, and induction of an enhanced innate response in the area where the antigen is present[22]. It is through the adaptive immune response that the immune system gains the ability to recognize a pathogen, and to mount an even stronger attack each time the pathogen is encountered, thereby preventing disease caused by that specific pathogen. T cells can be divided into CD8 expressing and CD4 expressing cells, of which the CD8 positive cells are the cytotoxic cells that kill the antigen bearing cells infected by viruses or other intracellular organisms. Upon activation, CD4 positive cells may differentiate into T helper (Th) cells or regulatory T (Treg) cells[29]. The activation of the Th cell causes it to promote various aspects of

the immune response, including immunoglobulin isotype switching and affinity maturation of the antibody response, macrophage activation, and enhanced activity of NK cells and cytotoxic T cells. Th1 cells are primarily involved in the activation of macrophages, NK cells and cytotoxic T cells, whereas B cell proliferation and production of antibodies are regulated by Th2 cells[30]. Treg cells limit and suppress the immune system and may control excessive immune responses to self antigens; an important mechanism in controlling the development of autoimmune diseases such as atherosclerosis[31].

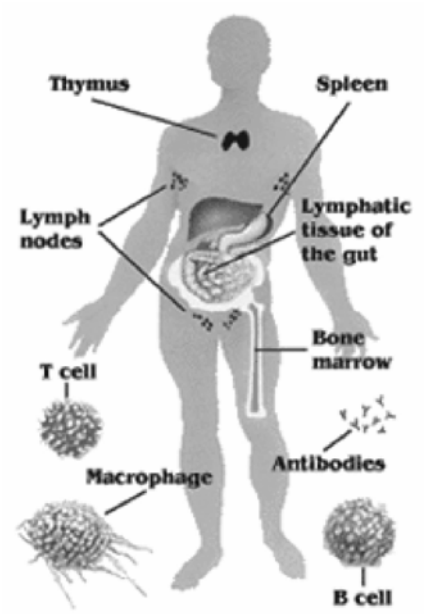

Fig. 2: Schematic overview of the immune system

Cytokines

Cytokines, small secreted proteins, are critical for the development and functioning of the immune system and play a major role in adaptive and innate immune responses and hematopoiesis[32,33]. In response to an antigen, virtually all cells of the immune systems, but especially Th cells and macrophages, produce cytokines that function as chemical messengers. Nowadays, more than 50 cytokines are clustered into several classes: interleukins (IL), tumor necrosis factors (TNF), interferons (IFN), CSFs, transforming growth factors (TGF) and chemokines[34,35]. Cytokines are pleiotropic, redundant, and multifunctional and can work either antagonistically or synergistically. They generally act at very low concentrations over short distances and short time spans in an autocrine or paracrine manner, but in some instances they work in an endocrine manner once they have entered the bloodstream. They bind to specific membrane receptors, which signal the cell via second messengers to proliferate, secrete effector molecules or alter gene expression of membrane receptors including cytokine receptors. Th1 cells primarily secrete IFN-γ, IL-2 and TNF-α, which promote cellular immunity against intracellular bacteria and

viruses. However, Th2 cells secrete a different set of cytokines, primarily IL-4, IL-10 and IL-13, which promote humoral immunity and immunity against extracellular parasites[30,36]. Furthermore, Th1 cytokines are generally referred to as pro-inflammatory and Th2 cytokines as anti-inflammatory. Common human diseases such as atopy/allergy, autoimmunity, chronic infections and sepsis are characterized by a dysregulation of the pro- versus anti-inflammatory and Th1 versus Th2 cytokine balance.

ATHEROSCLEROSIS

Despite significant progress in the management of atherosclerosis and its complications, cardiovascular disease remains the major cause of death in the Western world. Atherosclerosis is a progressive disease involving the development of vascular atherosclerotic lesions characterized by lipid accumulation, inflammation, cell death and fibrosis[23,24,37,38]. Atherosclerotic lesions can cause flow limiting stenosis leading to lack of oxygen and nutrition supply in the tissues located distally from the plaque. However, the most sever clinical events follow the rupture of the lesion, which exposes the pro-thrombotic material in the plaque to the blood and causes sudden thrombotic occlusion of the artery. In the heart, atherosclerosis can lead to myocardial infarction and heart failure, whereas in the brain, it can cause ischemic stroke and in peripheral tissues, it can result in renal impairment, hypertension, aneurysms and critical limb ischemia[37-39]. Although these clinical complications of atherosclerosis usually occur in the middle aged to elderly population, initiation of atherosclerotic lesion development is thought to start already in childhood. Epidemiological studies have identified numerous environmental and genetic risk factors such as hyperlipidemia, hypertension, diabetes mellitus, obesity, male sex, smoking, age, family history, physical inactivity and infections[38,40]. Furthermore, the prevalence of atherosclerosis is increasing all over the world due to the adoption of Western lifestyle and is likely to reach epidemic proportions in the coming decades.

Endothelial activation

The endothelium is a thin monocellular layer that covers all the inner surface of the blood vessels, separating the circulating blood from the tissues[41]. Under normal conditions, endothelial cells play a pivotal role in maintaining vessel wall homeostasis by producing vasoactive anti-inflammatory, anti-thrombotic, and cytostatic agents that help maintain vessel tone and protect the vessel wall against inflammatory cell and platelet adhesion, thrombus formation, and vascular cell proliferation[42-44]. The initial step in the development of atherosclerosis is now generally believed to result from an increase of the adhesiveness of the endothelium with respect to leukocytes or platelets, as well as its permeability to lipoproteins and its production of vasoactive molecules, cytokines, and growth factors[41-44]. Triggers of atherosclerosis, such as consuming a high-saturated-fat diet, smoking, hypertension, hyperglycemia, obesity, or insulin resistance can induce an enhanced expression of adhesion molecules such as intercellular adhesion molecule (ICAM)-1, vascular cell adhesion

molecule (VCAM)-1, P-selectin and E-selectin resulting in an increased adhesiveness of the endothelium[45]. Dysfunctional endothelium is the place where infiltration and accumulation of LDL into the vascular wall occurs and this accumulation is higher, when plasma LDL levels are elevated. Once infiltrated into the vascular wall, the native LDL becomes trapped and it undergoes enzymatic and non-enzymatic modification, including oxidation, lipolysis, proteolysis and aggregation[38]. Vascular endothelial cells are stimulated by accumulated modified LDL to produce a number of pro-inflammatory molecules, including adhesion molecules, chemotactic proteins such as monocyte chemotactic protein (MCP)-1 and growth factors such as macrophage colony-stimulating factor (M-CSF)[46,47] (**Fig. 3**).

Monocyte infiltration and differentiation

Upon activation of the endothelium, monocytes are recruited. Selectins on endothelial cells interact with their ligands on monocytes resulting in the rolling and tethering of monocytes on the vascular wall[48]. In addition, endothelial ICAM-1 and VCAM-1, as well as some integrins, induce firm adhesion of inflammatory cells at the vascular endothelium[48]. Next, monocytes migrate into the sub-endothelial space of the vascular wall, which is mediated by modified LDL and chemoattractant molecules such as MCP-1 and its receptor C-C chemokine receptor (CCR)-2[49-51]. Stimulated by M-CSF, infiltrated monocytes proliferate and differentiate into macrophages, which are antigen-presenting cells that scavenge lipoproteins and other extracellular debris, generate and degrade lipoproteins and produce inflammatory mediators like cytokines and extra-cellular matrix degrading enzymes[52] (**Fig. 3**).

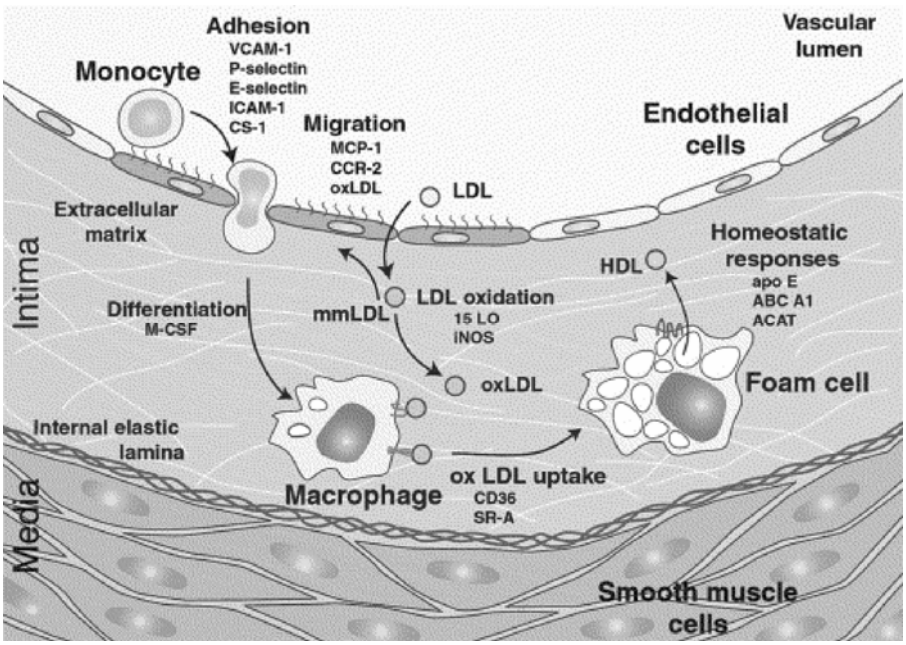

Fig. 3: Atherosclerotic lesion initiation (adapted from Glass and Witztum)[39]

Foam cell formation

In atherogenesis, monocyte derived macrophages react to the lesion microenvironment by internalizing and metabolizing a variety of components amongst which modified lipoproteins[53]. Excessive accumulation of CE from modified lipoproteins leads to the formation of foam cells. In vitro studies have shown that β-VLDL, a lipoprotein naturally induced by cholesterol-rich feeding of animals[54,55], and experimentally modified lipoproteins such as acetylated low-density lipoprotein (acLDL) or oxidized LDL (oxLDL)[56,57], are among the most potent inducers of foam cell formation. Uptake of CE from every type of lipoprotein is mediated by a different set of receptors.

The classical LDLr plays a crucial role in the uptake of LDL and its precursors, but its expression in macrophages is inhibited by the accumulation of cholesterol within the cell via a negative feedback mechanism[58]. However, studies with mouse peritoneal macrophages have shown that LDLr is the primary route for β-VLDL internalization[59]. Other members of the LDLr family are LRP-1 and the VLDL receptor (VLDLr), which are also involved in the cellular uptake of lipoproteins and lipoprotein remnants, such as β-VLDL[60,61]. In addition to the LDLr family, members of the scavenger receptor family mediate lipoprotein uptake[8]. Scavenger receptors recognize polyanionic macromolecules, including modified forms of LDL (such as oxLDL and acLDL) and have a physiological function in recognition and clearance of pathogens and apoptotic cells[62,63]. The family of scavenger receptors consists of several classes containing membrane bound proteins with widely different structures of which scavenger receptor class A (SR-A) and CD36 together account for up to 90% of total macrophage uptake of both AcLDL and OxLDL, as shown by Kunjathoor *et al* using transgenic mice lacking both SR-A and CD36[64]. However, other studies showed that aggregated LDL and VLDL can cause abundant foam cell formation in macrophages deficient in SR-A or CD36[65,66]. Scavenger receptor class B, type I (SR-BI), a CD36-related scavenger receptor, facilitates cholesterol efflux from macrophages to HDL[67], which delivers the cholesterol to the liver for further removal from the body, a process called reverse cholesterol transport. In addition, SR-BI can bind typical scavenger receptor ligands, including apoptotic cells, anionic phospholipids and lipoproteins, such as β-VLDL, oxLDL and acLDL, suggesting that SR-BI also mediates cholesterol uptake in macrophages[68,69]. This dual role of SR-BI in foam cell formation is underlined by the findings that macrophage SR-BI is either pro-atherogenic (small fatty streak) or anti-atherogenic (advanced lesion), depending on the stage of lesion development[70]. Other scavenger receptors, such as CD68, lectin-like oxidized LDL receptor (LOX)-1, and SR-phosphatidylserine and oxidized lipoprotein (SR-PSOX) are able to bind oxLDL[71]. However the roles of these and other scavenger receptors, including macrophage receptor with collagenous structure (MARCO), in atherogenesis remain to be determined.

Most lipoproteins taken up by macrophages are finally transported towards lysosomes, where they are degraded into amino acids and free cholesterol. When released into the cytosol, free cholesterol is re-esterified by acyl CoA:cholesterol acyltransferase (ACAT)-1 for storage in lipid droplets that characterize foam cells[72]. Cholesterol efflux from the macrophage/foam cell is another important process in the development of foam cell formation,

because foam cell formation is the result of an imbalance in cholesterol homeostasis[52]. ABCG1[12,73,74] and ABCA1[75,76] are the key mediators of cholesterol efflux to HDL and apoA-I, respectively. As mentioned above, SR-BI is also able to facilitate cholesterol efflux to HDL.
The transformation of macrophages into foam cells in atherosclerotic lesions can be affected by a variety of factors, including inflammatory mediators and nuclear receptors via enhancing or inhibiting the expression of the genes involved in cholesterol uptake and/or efflux. The accumulation of foam cells and intercellular lipid in the vascular wall characterize a fatty streak, which does not lead to significant obstruction of the arterial lumen. Although macrophage derived foam cells outnumber other cell types in fatty streaks, T cells are also present in these early lesions[39] (**Fig. 3**).

Advanced lesions and rupture
Although not clinically significant in themselves, fatty streaks can evolve into more complex lesions. Lesion progression (**Fig. 4**) involves the influx of T cells, which elaborate cytokines that influence the functional properties of nearby endothelial cells, macrophages, and smooth muscle cells. Smooth muscle cells migrate from the media into the intima, where they accumulate cholesterol and become smooth muscle cell-derived foam cells[39]. The death of foam cells is accompanied by the extracellular accumulation of lipids and cellular debris leading to the formation of a necrotic core that becomes covered by a fibrous cap consisting of smooth muscle cells and a collagen rich extracellular matrix[77]. In addition, other cell types are present in advanced lesions including dendritic cells, mast cells, B cells, natural killer (NK) cells and NKT cells, as reviewed by VanderLaan and Reardon[78].

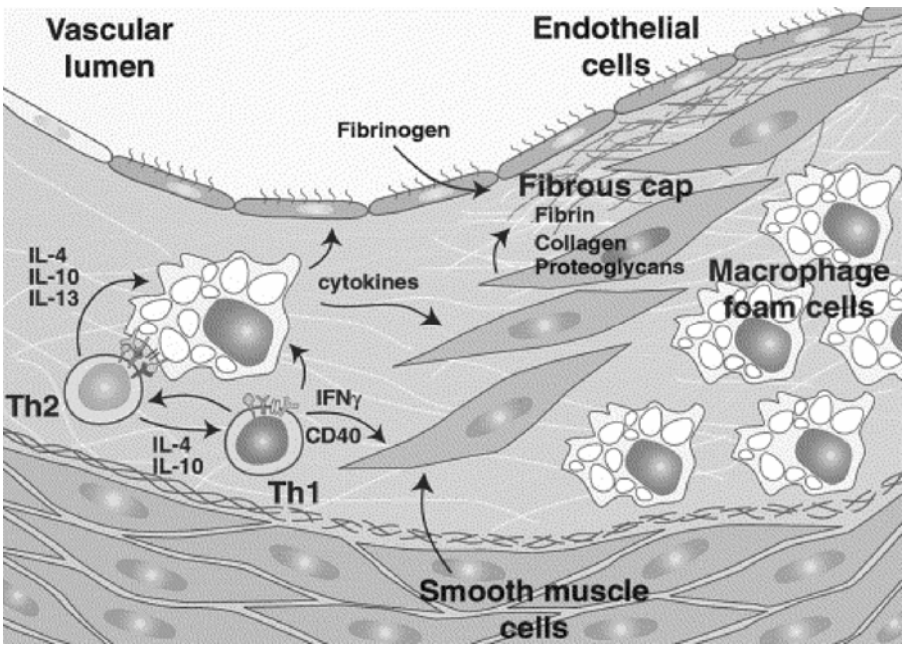

Fig. 4: Atherosclerotic lesion progression (adapted from Glass and Witztum)[39]

At this stage, the vascular wall can often enlarge and compensate for the developing plaque via outward remodeling, thereby preventing severe narrowing of the vessel and preserving the flow of blood[79]. As the lesion grows, increasing numbers of macrophage foam cells accumulate around the necrotic core and in adjacent areas representing lesion shoulders[38,39]. Around the necrotic core and in shoulder areas, macrophages and macrophage derived foam cells produce and release matrix metalloproteinases (MMP) and other proteolytic enzymes, which cause degradation of the matrix[80-82]. In addition, activated macrophages can induce apoptosis in smooth muscle cells resulting in a shortage of collagen[83]. The simultaneous production of MMPs and reduction of collagen affect the thickness of the fibrous cap in a negative way[84]. Rupture of the fibrous cap exposes the content of the lipid core to the blood, initiating coagulation, the recruitment of platelets and the formation of a thrombus, which causes most acute coronary syndromes[39] (**Fig. 5**).

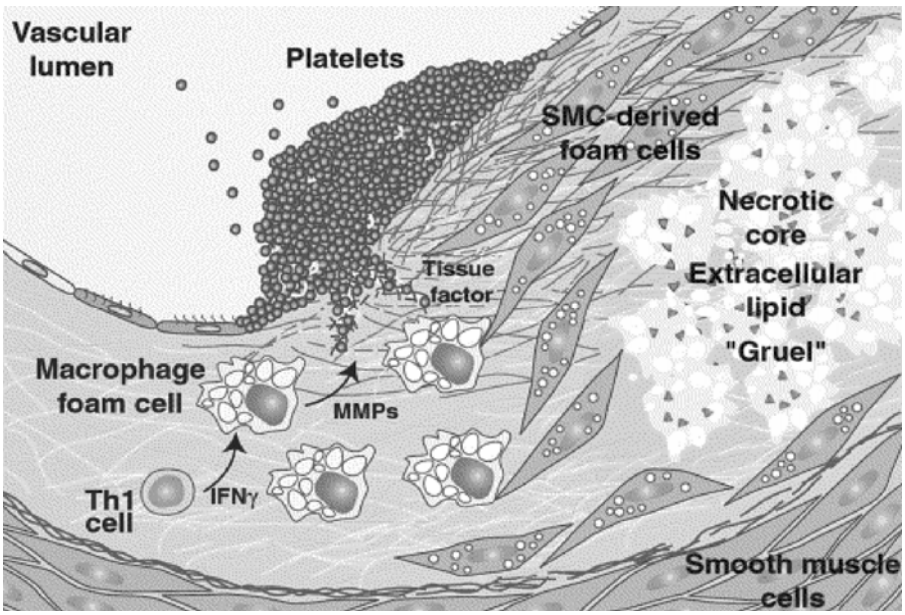

Fig. 5: Lesion rupture and thrombosis (adapted from Glass and Witztum)[39]

INFLAMMATION IN ATHEROSCLEROSIS

Inflammation is involved in all stages of atherosclerosis from its initiation to the thrombus formation causing its clinical complications (**Fig. 6**). One of the triggers of atherosclerosis is the modification of trapped LDL in the vascular wall leading to an enhanced expression of adhesion molecules by endothelial cells, thereby allowing the attachment of leukocytes to the vascular wall[85]. The importance of adhesion molecules in the initiation of atherosclerosis is shown by animal studies in which mice deficient in E-selectin and P-selectin[86] and mice expressing defective VCAM-1[87] develop

less severe atherosclerosis. Many cytokines, including IL-1β, TNF-α, and IFN-γ, have been implicated in the induction of adhesion molecules and chemokines in the vascular wall[88,89]. Cytokines also play an important role in the induction of chemokines, particularly IL-8 and MCP-1 that are involved in monocyte adhesion and migration into the vascular wall[90]. Under the influence of M-CSF, infiltrated monocytes differentiate into macrophages and start to express scavenger receptors and produce cytokines[91,92]. In several mouse models, M-CSF deficiency resulted in a dramatically reduced atherosclerotic lesion development[93,94]. Although the uptake of modified lipoproteins by scavenger receptors is thought to be central to foam cell formation, it also stimulates the pro-inflammatory phenotype of macrophages[71]. In addition, uptake by scavenger receptors can also lead to MHC restricted antigen presentation of the internalized material to T cells, thereby activating an adaptive immune response[95,96]. Cytokines greatly affect the expression of scavenger receptors and other key players in foam cell formation. IFN-γ and TNF-α are examples of cytokines that stimulate foam cell formation either by upregulation of receptors for uptake of modified lipoproteins alone (TNF-α)[97,98] or in combination with inhibiting cholesterol efflux (IFN-γ)[99,100]. In contrast, TGFβ1 is able to inhibit foam cell formation via inhibition of receptors for lipoprotein uptake and enhancement of macrophage cholesterol efflux[101]. Moreover, TGFβ1 is able to inhibit downregulation of ABCA1 expression and macrophage cholesterol efflux induced by IFN-γ[102]. However, IL-10, another anti-inflammatory cytokine, enhanced oxLDL induced foam cell formation by anti-apoptotic mechanisms[103].

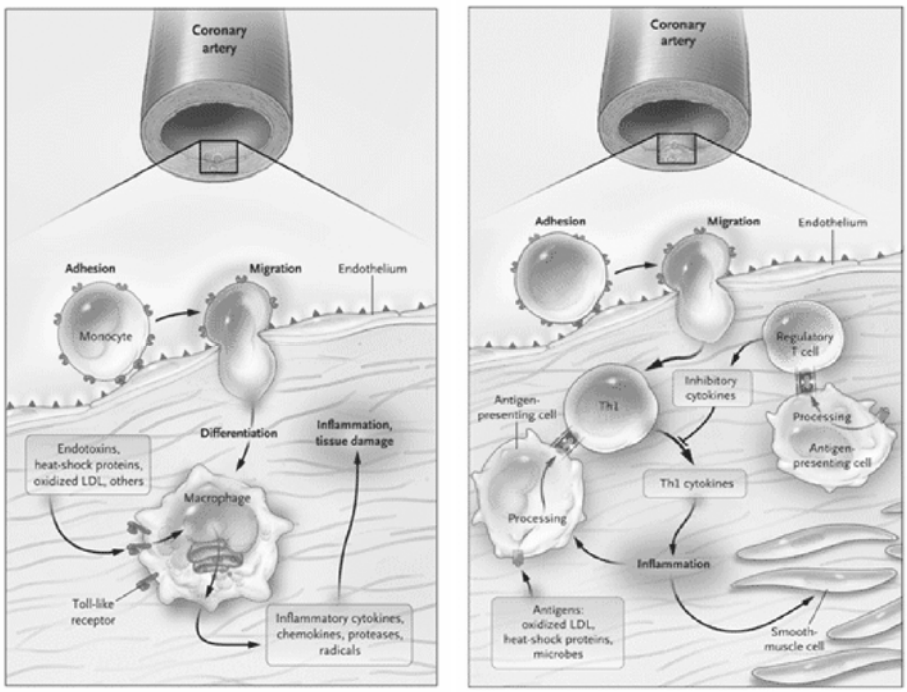

Fig. 6: Macrophages and T cells in lesion inflammation (adapted from Hansson)[23]

Progression from a fatty streak to a more complex lesion is characterized by proliferation and migration of smooth muscle cells toward the intima and collagen synthesis. Accumulation of inflammatory and vascular smooth muscle cells, collagen, and lipid content results in growth of the lesion[37-39]. Several studies showed that inhibiting signaling of the immune mediators CD40 / CD40 ligand not only prevented initiation of atherosclerosis, but also progression of existing lesions[104,105]. Genetic studies show that the immunological receptor-ligand pair OX40 / OX40 ligand, which enhances the proliferation and differentiation of T lymphocytes and contributes to ongoing Th1 or Th2 responses, is linked to atherosclerosis susceptibility and myocardial infarction[106,107]. Continued release of cytokines in the lesion by macrophages and T cells not only perpetuates inflammation within the lesion but also modulates smooth muscle cell activity[108]. For example, cytokines such as TNF-α and IFN-γ can promote the uptake of modified lipoproteins that leads to smooth muscle cell derived foam cells *in vitro*[109] and IL-10 inhibits intimal smooth muscle cell accumulation in several animal models[110,111]. Acute coronary syndromes often result from rupture of lesions, usually at sites with a thin fibrous cap[112]. One of the major constituents of the fibrous cap is collagen produced mostly by smooth muscle cells. TGFβ1 stimulates collagen production, whereas IFN-γ produced by T cells in the lesion inhibits both basal and TGFβ1 induced collagen production[113]. Collagen in the fibrous cap can be degraded by proteolytic enzymes, including MMPs. TNF-α, CD40L and IL-1 stimulate macrophages to produce MMPs [114-116], but the anti-inflammatory IL-10 and TGFβ1 inhibit MMPs[117,118]. TGFβ1, however, contributes to restenosis[119] and is recently identified as a crucial factor for the differentiation of the novel, highly inflammatory Th17 cell sub set[120], indicating a more complex role for TGFβ1 in atherosclerosis. The thrombogenicity of the lesion is also affected by inflammation. Macrophage production of tissue factor, which initiates the coagulation cascade once exposed to factor VII in the blood, is stimulated by TNF-α[121] and CD40 ligand expressed by T cells[122]. Furthermore, platelet production and reactivity is affected by cytokines, such as IL-6[33,121].

In atherosclerotic lesions, cytokines that promote Th1 differentiation, such as IL-12 and IL-18, are produced[33,123]. Consequently, Th1 cytokines, including IFN-γ, IL-2 and TNF-α, are produced and activate macrophages and other cells in the lesion to secrete pro-inflammatory cytokines[124]. Studies have clearly shown a critical pathogenic role for the Th1 response in atherosclerosis at the cell-type level (transfer of Th1 cells), the cytokine production level (IL-12, IL-18, and IFN-γ) and even at the level of Th1 cell commitment[33]. In contrast, a Th2 immune response is suggested to counteract the Th1 promoted atherosclerosis. Studies in which the Th2 cytokine IL-10 protect against atherosclerosis underline this hypothesis[125,126]. However, deficiency in IL-4, a prototypic Th2 cytokine, has been associated with decreased lesion formation and progression[127,128], suggesting that inducing a Th2 response is not always beneficial in atherosclerosis (**Fig. 7**).

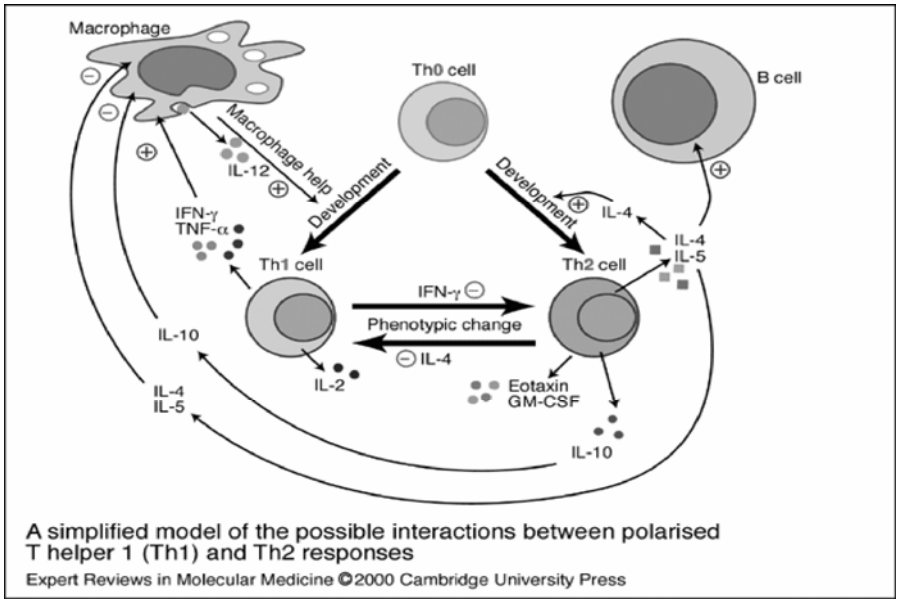

Fig. 7: Interactions of Th1 and Th2 cells (adapted from Harper *et al*)[129]

In addition to macrophage foam cells and T helper cells, other inflammatory cell types have been implicated in atherosclerosis[78]. Recently, it was shown in several mouse models that naturally arising Treg cells, which actively maintain immunological tolerance to self and non-self, including transplantation and food related, antigens, are powerful inhibitors of atherosclerosis[130]. In addition, recent experimental evidence, reviewed by Whitman and Ramsamy[131], suggests that although NK cells and NKT cells are not sufficient to cause atherosclerosis, they do play an important role in accelerating lesion development by modulating the function of other more prominent immune cells found within the developing atherosclerotic lesion such as conventional T cells and macrophages. Elimination of the entire B cell population, genetically or via splenectomy, increases atherosclerosis[132,133]. A specific subtype of B cells, B-1 cells, produces natural antibodies against modified self-antigens, such as oxLDL. Interestingly, antibodies that recognize oxLDL have been found in the circulation of both humans and mice[134]. Although B cells have been found in the adventitia[135], they are rarely detected in lesions. Mast cells were found in the shoulder region of atherosclerotic lesions and in the adventitia[136,137]. Upon activation, mast cells release the contents of their large cytoplasmic granules that contain vasoactive substances, proteolytic enzymes, pro-inflammatory cytokines and growth factors[138], indicating that activation of these cells may aggravate atherosclerosis. Dendritic cells are efficient antigen presenting cells that are present in vessels before atherosclerosis[139], but they increase in number and become activated during lesion development[140], where they colocalize with T cells in the shoulder region[141]. Normally, activated dendritic cells migrate to secondary lymphoid tissues and present self peptides to naïve T cells in order to induce tolerance. However, hyperlipidemia, which is associated with atherosclerosis, suppresses the migration of skin dendritic cells[142],

suggesting an impaired efflux of activated dendritic cells from atherosclerotic lesions. Inhibition of the migration of activated dendritic cells results in the loss of local tolerance and thereby in the triggering of local inflammation, an influx of inflammatory cells and the production of inflammatory cytokines. Neutrophils are primarily involved in acute inflammation via engulfing damaged tissue and bacteria, killing invading microbes and secreting proteolytic enzymes[143]. Strikingly, these major cells of the innate immune response are not present in early and stable atherosclerotic lesions. They have been found in eroded and ruptured lesions[144], but it has to be investigated whether they cause erosion and rupture by secreting proteinases or whether they are attracted to the site of injury.

With respect to the importance of inflammation in atherosclerosis, many have hypothesized that infectious agents might be the cause of chronic inflammation in lesions. Specific organisms that have been implicated include *Chlamydia pneumoniae*, herpes viruses and *Helicobacter pylori*[145-147]. Serum antibodies against these pathogens have been associated with atherosclerosis[147] and cytomegalovirus, *Chlamydia pneumoniae* and many other bacteria have been detected in human atherosclerotic lesions[145,146]. Recently, the total pathogen burden concept has suggested that while a single pathogen contributes only slightly to the pathogenesis of atherosclerosis, the cumulative effects of infectious agents contribute greatly. However, many studies over the years resulted in conflicting data about a direct causal relation between the pathogens and atherosclerosis. Although it remains unclear if pathogens are etiological factors in atherosclerosis, they can aggravate the inflammatory process in atherosclerosis. For instance, *Chlamydia pneumoniae* can infect endothelial cells resulting in an enhanced expression of adhesion molecules and it stimulates the production of cytokines and MMPs in macrophages[148,149]. Furthermore, *in vitro* infection of macrophages with cytomegalovirus increases secretion of IL-1, TNF-α and M-CSF.

Results of clinical trials investigating anti-chlamydial antibiotics as an addition to standard therapy in patients with coronary artery disease have been inconsistent[150-152]. Therefore, Andraws *et al* conducted a meta-analysis of these clinical trials and found that evidence available to date does not demonstrate an overall benefit of antibiotic therapy in reducing mortality or cardiovascular events in patients with coronary artery disease[153].

Lipopolysaccharides

Lipopolysaccharide (LPS) is a major constituent of the outer membrane of Gram-negative bacteria and, when released from bacteria, is one of the most potent inducers of inflammation[154-157]. LPS is composed of three structural elements: a highly variable outer O-antigen oligosaccharide, a more conserved core oligosaccharide and a lipid A component, which is responsible for the pro-inflammatory properties of LPS[158]. The first host protein involved in the recognition of LPS is LPS-binding protein (LBP), which is an acute-phase protein that transfers LPS to the cell surface by binding to it and catalyzing complex formation of LPS with the LPS receptor molecule CD14[159]. Formation of the complex between LPS and CD14,

either soluble or membrane bound, facilitates the binding of LPS to the LPS receptor complex composed of TLR4 and MD2[160]. In addition, Triantafilou *et al* have proposed that other molecules may be involved in LPS recognition including heat shock proteins, chemokine receptor 4, growth differentiation factor 5, CD11b/CD18 and CD81[161]. The TLR4 signaling cascade following LPS binding is enhanced by homodimerization of the receptor and subsequent recruitment of adaptor molecules, such as myeloid differentiation factor 88[162]. The main players involved in eliciting the functional effects of LPS are nuclear factor κB (NFκB), mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/Akt pathways, of which NFκB is the major one[163]. In monocytes and macrophages, these transcription factors regulate the production of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6, which serve as endogenous mediators of inflammation through interactions with various target cells[156].
In addition, LPS affects many cells and processes in atherosclerosis. LPS activates the endothelium directly via inducing the production of pro-inflammatory mediators, such as IL-6, IL-8 and MCP-1[164], via increasing expression of the adhesion molecules E-selectin, ICAM-1 and VCAM-1[165] and via enhancing expression of tissue factor[166]. Endothelial as well as monocyte activation induced by LPS results in a higher monocyte binding to the endothelium[167,168]. Besides enhancing the macrophage production of pro-inflammatory cytokines, LPS induces foam cell formation via affecting the expression of many genes involved in foam cell formation, such as LDLr, LRP-1, SR-BI and ABCA1[169-172]. Furthermore, LPS reduces collagen production and increases MMP secretion[173], indicating that LPS makes the atherosclerotic lesion more vulnerable to rupture.

Interleukin-9
IL-9 is a pleiotropic cytokine that was first identified as P40, a T cell growth factor[174,175]. However, this cytokine composed of 144 amino acid also affects many other cells, including mast cells, B cells, lung epithelial cells and macrophages[176-178]. Th2 cells are the major source of IL-9 and after its secretion, IL-9 binds to the IL-9 receptor (IL-9R), which is a member of hematopoietin receptor superfamily consisting of a common γ_c subchain and a specific IL-9Rα chain[179]. Upon binding to its receptor, IL-9 exerts its effects mainly via the Janus kinase/Stat (JAK/Stat) pathway[180].
Increased IL-9 production seems to be implicated in major pathologies such as asthma and tumorigenesis, especially of lymphomas. Localization of the IL-9 gene, together with many other Th2 cytokines and asthma related genes, on human 5q31-35 made it a candidate gene for asthma[181]. This hypothesis was underlined by many studies in which IL-9 affected mucus and chemokine production in pulmonary epithelium, IgE production, mast cell differentiation, bronchial hyperresponsiveness and eosinophil activation[182]. Furthermore, transgenic mice overexpressing IL-9 present features of asthma[183,184] and treatment with an IL-9 neutralizing antibody inhibits the development of allergic pulmonary inflammation and airway hyperresponsiveness[185,186]. In contrast, a Th2 response to allergen after sensitization as well as other features of asthma are still present in IL-9 deficient mice[187], suggesting that IL-9 may enhance asthma but is not

mandatory for its development. IL-9 overexpression induces thymic lymphomas in mice[188] and IL-9 production is associated with Hodgkin and NKT cell lymphomas, possibly via an autocrine loop[189,190]. Furthermore, thymic T cell lymphomas are protected from dexamethasone induced apoptosis by IL-9[191]. In addition, IL-9 is a susceptibility factor in Leishmania major infection[192] and leads to an early death in mice with chronic Schistosoma mansoni infection[193] by inducing a Th2 response.
On the other hand, several studies show that IL-9 may exhibit protective effects by inhibiting inflammatory responses. IL-9 shows these anti-inflammatory effects in the protection of mice from Gram-negative bacterial shock by suppression of TNF-α, IL-12, and IFN-γ, and induction of IL-10[194]. Furthermore, IL-9 may directly deactivate LPS stimulated blood mononuclear phagocytes via induction of TGFβ1 production by these cells[195]. Other studies revealed a protective role for IL-9 in the host immunity to intestinal nematode infections, such as *Trichuris muris*[196,197]. Interestingly, IL-9 induces expression of three intracellular cytokine signal inhibitors: cytokine-inducible SH2-containing protein, suppressor of cytokine signaling (SOCS)-2 and SOCS-3[198], which negatively regulate signaling of many pro-inflammatory and pro-atherogenic cytokines. A recent study of Lu *et al*, unexpectedly, indicated that Treg cells produce IL-9 to recruit and activate mast cells, which appeared to be crucial in allograft tolerance[199]. Despite its anti-inflammatory functions, the role of IL-9 in autoimmune diseases has not been investigated yet.

Interleukin-10
IL-10 was originally described as cytokine synthesis inhibitory factor that was produced by Th2 cells and inhibited Th1 cytokine production[200]. However, after extensive research, IL-10 is now regarded as a pleiotropic cytokine, which is produced by various cell populations, including T cell subsets, B cells, monocytes, and macrophages[201]. After homodimerization of IL-10, its activity is mediated by its specific cell surface receptor complex, which is expressed on a variety of cells, in particular immune cells. As reviewed by Moore *et al*, IL-10 signaling is mediated by JAK/STAT and involves inhibition of the NFκB signaling pathway[202]. Functionally, IL-10 acts on a wide variety of cells, including B cells, NK cells, cytotoxic T cells, Th cells, mast cells, granulocytes, dendritic cells, epithelial cells and endothelial cells[201-204]. Because IL-10 affects a broad spectrum of cells, it is involved in the pathogenesis of many diseases, such as cancer, viral infections and autoimmune diseases[201-204]. Although IL-10 is commonly regarded as an anti-inflammatory, immunosuppressive cytokine, evidence is accumulating that IL-10 also possesses some immunostimulating properties. It suppresses inflammation associated immune responses (Th1, antigen presentation, pro-inflammatory cytokine secretion by macrophages, modulation of Th2), but stimulates functions of innate immunity (NK cell activity, non-inflammatory phagocytosis) and of Th2 related immunity both directly and indirectly[201].
Almost 10 years ago, IL-10 expression was found in atherosclerotic lesions[123,205], after which the role of IL-10 in the development of atherosclerosis was extensively investigated. Overexpression of IL-10 resulted in attenuation of atherogenesis, suggesting a protective role for

endogenous IL-10[125,206,207]. This effect was underlined by studies in IL-10 deficient mice that showed an enhanced atherosclerotic lesion development[126,208]. IL-10 prevented induced endothelial ICAM-1 and VCAM-1[209] and monocyte CD18 and CD62L expression[210], thereby inhibiting monocyte binding and infiltration to the vessel wall. Moreover, IL-10 decreases MMP-9 production and activity and simultaneously induces expression of tissue inhibitor of metalloproteinases (TIMP)-1 in macrophages[211]. Other protective effects of IL-10 are the inhibition of macrophage production of many pro-inflammatory cytokines and induction of a shift in the Th balance to a Th2 profile[200,212,213].
Recently, Rubic *et al* proposed that reduced CD36 expression and oxLDL uptake in combination with enhanced ABCA1 and ABCG1 expression and cholesterol efflux could attribute to the anti-atherosclerotic actions of IL-10[214]. On the other hand, Halvorsen *et al* suggested that IL-10 enhanced oxLDL uptake, at least partly by counteracting apoptosis induced by oxLDL[103]. However, both studies showed only moderate differences in oxLDL induced lipid uptake making it difficult to draw conclusions about the role of IL-10 in foam cell formation, whereas the effects on apoptosis were more profound.
In addition, several animal studies showed that IL-10 influences lipid metabolism resulting in lower serum total cholesterol levels[125,207]. However, the mechanism of the serum cholesterol lowering effect of IL-10 is not yet clarified.

THESIS OUTLINE

The main aim of this thesis was to investigate interactions between lipids, inflammation and atherosclerosis. Although atherosclerosis is considered as a chronic inflammatory disease and IL-9 affects many inflammatory processes, the role of IL-9 in atherosclerosis has not been elucidated yet. Therefore, in **chapter 2**, we investigated the effect of IL-9 treatment and of vaccination against endogenous IL-9 on atherosclerotic lesion development in LDLr deficient mice, a well established mouse model for atherosclerosis. Foam cell formation is a critical process in atherogenesis and is affected by inflammatory mediators. We investigated whether IL-9 affected β-VLDL induced foam cell formation using RAW 264.7 murine macrophage cells in **chapter 3**. Inflammatory mediators regulate the expression of many genes involved in foam cell formation and lipid loading of macrophages, in turn, changes the response of the macrophages to inflammatory mediators. In **chapter 4**, we loaded RAW cells with β-VLDL to determine the effect of foam cell formation on the response of macrophages to LPS with a specific emphasis on the expression of lipid related genes. In addition to TLR4, the signaling receptor for LPS, several scavenger receptors mediate binding and internalization of LPS, thereby neutralizing LPS. In **chapter 5**, we set out to examine the role of one of these scavenger receptors, SR-BI, in the response to LPS using SR-BI wild-type and SR-BI deficient mice.
Uptake of dietary lipids contributes to cholesterol homeostasis, in which the liver and especially liver parenchymal cells are key players. Microarray analysis was used to determine the effect of Western-type diet feeding of

LDLr deficient mice on liver parenchymal cells in **chapter 6**. Although serum IL-10 levels are negatively correlated with cholesterol levels in several studies, no explanation for this effect has been described. In **chapter 7**, the influence of adenoviral IL-10 treatment on liver parenchymal cells of LDLr deficient mice, which were fed a Western-type diet, was determined using microarray analysis.

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