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Immunomodulation and its Effector Mechanisms in Atherosclerosis

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To my parents - *For encouraging me to become myself*

To Mats - *For supporting me to stay myself*

ABSTRACT

Atherosclerosis is a disease of the arterial intima, characterized by cholesterol deposition, inflammation and fibrosis. The pathogenesis of atherosclerosis is related to both innate and adaptive immune responses, and modulation of immune reactions in animal models of atherosclerosis affects the progression of the disease. The studies in this thesis have utilized apolipoprotein E deficient (*Apoe*$^{-/-}$) mice to unravel immune mechanisms involved in progression and protection of atherosclerosis.

Apoe$^{-/-}$ mice and wild-type mice were compared to clarify the role of apolipoprotein E (apoE) as a modulator of immune responses. Our data show that apoE controls T cell activation by down-regulating the expression of MHC class II and costimulatory molecules on the antigen-presenting cell. We thereby explain how apoE can inhibit T cell proliferation.

To investigate the effect of Interleukin 18 (IL-18) on atherosclerosis in the absence of adaptive immunity, immunodeficient SCID/*Apoe*$^{-/-}$ mice were treated with IL-18. We demonstrate that IL-18 promotes atherogenesis in the absence of T cells and show that NK cells, macrophages and vascular cells produce IFNγ in response to IL-18 in amounts that are sufficient for disease progression.

Transfer of MDA-LDL specific CD4^{+} T cells to SCID/*Apoe*$^{-/-}$ mice was performed to determine whether the proatherogenic effect of CD4^{+} T cell transfer is antigen specific. The transfer accelerates atherosclerosis and leads to elevated levels of IFNγ, demonstrating that Th1 cellular immunity directed to MDA-LDL promotes atherosclerosis in hypercholesterolemic mice.

To explore the role of dendritic cells in atherosclerosis, we transferred DC loaded with MDA-LDL to *Apoe*$^{-/-}$ mice. This treatment induced antigen-specific pro-atherogenic immunity that augmented local inflammation and growth of atherosclerotic lesions, illustrating that DC may take part in the initiation of atherosclerosis-related immunity.

Finally, we studied immunization-induced atheroprotection in the absence of B cells and antibodies in μMT/*Apoe*$^{-/-}$ mice. Our data demonstrate B cell-independent protection by MDA-LDL immunization, paralleled by induction of regulatory T cells. We thereby confirm that immunization with MDA-LDL inhibits disease development in hypercholesterolemic mice and identify a B cell-independent protective mechanism.

In conclusion, we have studied immunomodulation and effector mechanisms in mouse models of atherosclerosis. In brief, we show that; apoE acts as a dampener on the antigen presenting cell, IL-18 is pro-atherogenic even in the absence of adaptive immunity, DC presenting MDA-LDL initiate pro-atherogenic reactions and protective immunization remains in B cell deficient mice.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Ateroskleros, eller åderförkalkning som sjukdomen kallas i dagligt tal, är en sjukdom som drabbar våra blodkärl. Redan när vi är unga kan man se tidiga tecken på ateroskleros i kärlen. Sjukdomen förvärras med åren och kan så småningom få allvarliga konsekvenser såsom hjärtinfarkt och stroke. En av de viktigaste riskfaktorerna för att drabbas av allvarlig ateroskleros är att ha höga halter av kolesterol i blodet. Kolesterol är nödvändigt för kroppen, men om vi har för mycket så ansamlas det i s.k. LDL-partiklar i blodet. LDL-partiklarna tränger in i blodkärlsväggen där de fastnar och blir oxiderade. Oxiderat LDL (oxLDL) angrips av immunförsvaret och det bildas en inflammation i kärlväggen som leder till fläckvisa förtjockningar och ibland till och med förhårdningar av kärlväggen. Dessa förändringar kallas aterosklerotiska plack. När kärlväggen blir tjockare blir kärlets inre diameter mindre och blodet får svårare att komma fram, vilket leder till att kroppens vävnader som försörjs med blod från det förträngda kärlet får dåligt med syre. Om man har otur kan insidan av den förtjockade kärlväggen spricka sönder. Detta gör att blodet levrar sig inuti blodkärlet och bildar en s.k. blodpropp och vävnaden som blir utan blod och syre kommer att dö. När en propp bildas i ett av kärlen i hjärtmuskeln får man en hjärtinfarkt, och om proppen sitter i ett av hjärnans blodkärl får man en stroke.

De studier som ingår i den här avhandlingen handlar om den inflammation som startas av oxLDL och som leder till att placken växer och till slut spricker. Vi har utfört flera olika experiment där vi har påverkat immunförsvaret på olika sätt för att studera vad effekterna blir på placken. För att göra dessa studier har vi använt genmodifierade möss som saknar en gen som heter apolipoprotein E (apoE). ApoE är viktigt för omsättningen av kolesterol i kroppen, och möss som saknar apoE får väldigt höga halter av kolesterol i blodet vilket gör att de utvecklar ateroskleros på samma sätt som människor.

I den första studien har vi jämfört immunförsvaret mellan möss som saknar apoE och vanliga möss. Andra forskare har tidigare visat att apoE kan dämpa immunreaktioner och vi har nu visat att detta sker genom att apoE dämpar vissa av de signaler som aktiverar immunförsvaret. Detta betyder att möss utan apoE inte bara har en störning i kolesterol-omsättningen utan även har ett överaktivt immunförsvar som bidrar till aterosklerosutvecklingen.

I den andra studien har vi studerat en av immunförsvarets signalmolekyler som heter interleukin-18 (IL-18). Detta är en molekyl som driver på inflammationen genom att aktivera immunförsvarets celler och därmed öka tillväxten av plack. Man har tidigare trott att IL-18 framför allt verkar genom att aktivera en viss typ av vita blodkroppar som kallas *hjälpar* T celler. Vi har dock visat att IL-18 ökar inflammationen och tillväxten av plack även i möss som inte har några T celler, och vi har därtill identifierat flera andra celltyper som reagerar på IL-18.

I vår tredje studie har vi undersökt betydelsen av *hjälpar* T celler i immunreaktionen mot oxLDL. Vi har visat att om man ger *hjälpar* T celler som är instruerade att angripa oxLDL till möss, så kommer deras plack att bli inflammerade och växa. Detta betyder alltså att *hjälpar* T celler som angriper oxLDL är en viktig orsak till ateroskleros.

I den fjärde studien har vi studerat betydelsen av dendritiska celler (DC) i ateroskleros. DC är viktiga för att sätta igång immunreaktionen och instruera immunförsvaret vad det är som behöver angripas. Man har tidigare sett DC i aterosklerotiska plack, men inte riktigt vetat om de har någon funktion i sjukdomen. I våra experiment har vi visat att om DC instruerar immunförsvaret att angripa oxLDL, kommer inflammationen i placken och tillväxten av dem att öka. Vi har därmed visat att DC kan bidra till ateroskleros.

Om man ger möss oxLDL blandat med en speciell substans som aktiverar immunförsvaret så får mössen konstigt nog ett skydd mot ateroskleros. Det är förstås väldigt intressant att ta reda på hur detta fungerar. Man har trott att en viss typ av vita blodkroppar, s.k. B celler, har varit inblandade i detta skydd, men vi har i vår femte studie visat att behandlingen ger skydd även i möss som inte har några B celler. Istället har vi identifierat en annan möjlig skyddsmekanism. Vi har nämligen sett att vita blodkroppar som har till uppgift att dämpa immunreaktioner, s.k. *regulatoriska* T celler, ökar efter behandlingen.

För att summera arbetet i denna avhandling, så har vi använt möss med ateroskleros för att studera immunförsvarets betydelse i sjukdomen. Vi har bidragit till kunskapen om vad som ökar och minskar ateroskleros genom att fylla i kunskapsluckor, utvidga koncept och introducera nya principer. I förlängningen kan denna kunskap leda till bättre metoder för att förebygga och behandla åderförkalkning.

LIST OF PUBLICATIONS

I. Apolipoprotein E modulates immune activation by acting on the antigen-presenting cell.
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Arterioscler Thromb Vasc Biol. 2006 Apr;26(4):864-70.

IV. Transfer of dendritic cells pulsed with modified low-density lipoprotein aggravates atherosclerosis.
Hjerpe C, Johansson D, Hansson GK, Zhou X.
Submitted

V. Atheroprotection by immunization remains in B cell deficient mice.
Hjerpe C, Johansson D, Robertson AK, Hansson GK, Zhou X
Submitted

CONTENTS

LIST OF ABBREVIATIONS

APC	Antigen-presenting cell/s
ApoB	Apolipoprotein B
ApoE	Apolipoprotein E
Apoe$^{-/-}$	Apolipoprotein E knockout
β2GPI	Beta-2 glycoprotein I
BCR	B cell receptor
CBA	Cytometric bead array
CD	Cluster of differentiation
CD40L	CD40 ligand
CFA	Complete Freund's adjuvant
Con A	Concanavalin A
Cpm	Counts per minute
CRP	C-reactive protein
CTL	Cytotoxic T lymphocyte/s
DC	Dendritic cell/s
DNA	Deoxyribonucleic acid
EC	Endothelial cell/s
ELISA	Enzyme-linked immunosorbent assay
GM-CSF	Granulocyte-monocyte colony-stimulating factor
HDL	High-density lipoprotein
HMG-CoA	3-hydrocyl-3-methylglutaryl coenzyme A
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HSP	Heat-shock protein
ICAM	Intercellular adhesion molecule
IFA	Incomplete Freund's adjuvant
IFNγ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
KLH	Keyhole-limpet hemocyanin
LDL	Low-density lipoprotein
LDL-IC	Low-density lipoprotein immunocomplex
Ldlr	Low-density lipoprotein receptor

Ldlr$^{-/-}$	Low-density lipoprotein receptor knockout
LPS	Lipopolysaccharide
MCP	Monocyte chemotactic protein
M-CSF	Macrophage colony-stimulating factor
MDA	Malondialdehyde
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
NF-κB	Nuclear factor kappa B
NK cell	Natural killer cell
NK T cell	Natural killer T cell
OD	Optical density
oxLDL	Oxidized LDL
PCR	Polymerase chain reaction
PRR	Pattern recognition receptor
Rag	Recombination-activating gene
SCID	Severe combined immunodeficiency
ScR	Scavenger receptor
SMC	Smooth muscle cell/s
SR-A	Scavenger receptor A
TCR	T cell receptor
TGF	Transforming growth factor
Th cell	T helper cell
TLR	Toll-like receptor
Tr1 cell	Regulatory T cell type 1
Treg	Regulatory T cell
TNFα	Tumor necrosis factor alpha
VCAM	Vascular cell adhesion molecule
VDC	Vascular dendritic cell
VLA	Very late antigen
VLDL	Very low-density lipoprotein

1 INTRODUCTION

Atherosclerosis is a complex and chronic inflammatory disease, characterized by focal thickenings of the innermost layer of the arterial wall, the intima. These thickenings are called atheromas or atherosclerotic plaques/lesions. The atheromas are composed of lipids, extracellular matrix, vascular smooth muscle cells, endothelial cells, immune cells and debris (figure 1). The characteristic cell type of the atherosclerotic lesion is the foam cell, which is a macrophage filled with lipid droplets in its cytoplasm, giving it a foamy appearance. The earliest lesion, called a fatty streak, can be found already in fetal life and may progress into a more advanced lesion or disappear with time [1]. All stages of lesions contain cells and components of the immune system, such as activated T cells, antibodies and mast cells, all contributing to the progression of the plaque. Acute clinical complications such as myocardial infarction and ischemic stroke are caused by the formation of a thrombus on the atherosclerotic plaque due to physical disruption of the plaque surface. The degree of inflammatory activity influences the morphologic instability of the plaque and therefore the risk that it will rupture and cause myocardial infarction or stroke. A plaque prone to rupture is characterized by a thin fibrous cap, a large necrotic core, few smooth muscle cells and many activated inflammatory cells. A more stable plaque exhibit a smaller necrotic core, thicker fibrous cap, larger proportion of smooth muscle cells and fewer inflammatory cells. Uncovering the mechanisms by which specific immune cells and components impact lesion formation and stability is valuable for providing insight into the pathogenesis of atherosclerosis and for developing novel therapeutic approaches.

This thesis is focused on the role of the immune system in atherosclerosis and how immunomodulation can impact the pathogenesis of atherosclerotic lesion formation. I have chosen to first give an overview of the immune system, to provide some background information to everyone reading this thesis. In the sections following the overview I describe the many different studies on immunomodulation which represent our current knowledge about the role of immune reactions in atherosclerosis. In the end I discuss and summarize my own projects in the light of that knowledge.

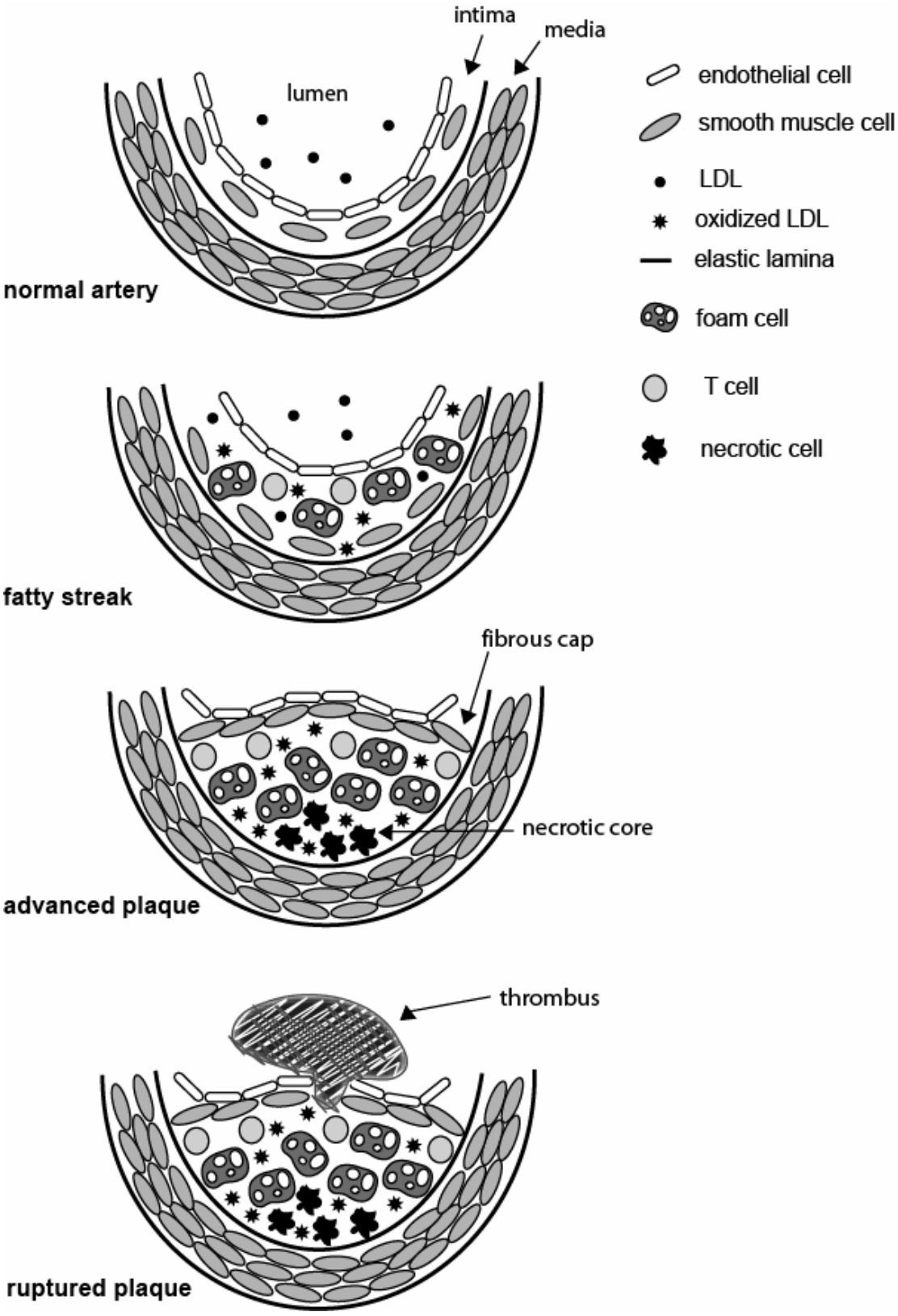

Figure 1. Stages of atherosclerosis development

1.1 OVERVIEW OF THE IMMUNE SYSTEM [2]

The function of our immune system is to protect us from infections. It consists of cells and molecules with remarkable abilities to distinguish self from non-self, and to eliminate foreign substances. The responses of the immune system to microbes or other foreign substances is referred to as *immune responses* and the protection achieved as *immunity*. The substance inducing the immune response is referred to as an *antigen*, recognized by the cells of the immune system by means of their surface receptors. The immune system is organized in two lines of defence; innate and adaptive immunity. The cells and molecules in innate and adaptive immunity co-operate efficiently and communicate by cell-to-cell contacts using surface molecules and by secreting soluble messengers called cytokines. The innate response stimulates and influences the nature of the adaptive response, and the adaptive response enhances the activities of the innate response.

1.1.1 Innate immunity

The components of innate immunity, also called native or natural immunity, is in place even before the infection, ensuring a rapid response. The function of the innate immunity is to prevent infection and eradicate microbes, and to send warning signals activating and influencing the adaptive immune response. The innate immunity recognizes structures that are common for groups of microbes via antigen receptors specific for certain patterns on the microbes, therefore called pattern recognition receptors (PRRs). These receptors are germline encoded and can bind about 10^3 different molecular patterns, leading to expression of inflammatory genes typical for innate immune responses. Scavenger receptors (ScRs) are one type of PRR, internalizing particles such as bacterial endotoxins and apoptotic fragments for destruction. Toll-like receptors (TLRs) are another type of PRR which are triggered by bacterial toxins, stress proteins and bacterial DNA. The innate defence is composed of epithelial barriers and circulating cells and proteins that recognize microbes or substances produced by microbial infections. The circulating components include for example phagocytic cells, natural killer cells, the complement system and opsonins.

1.1.1.1 Phagocytes

Neutrophils and macrophages are phagocytic cells with the task of identifying, ingesting and destroying microbes. Neutrophils are phagocytic cells involved in acute

inflammation by killing microbes and engulfing bacteria and damaged tissue. Macrophages are key players in innate immunity through their secretion of enzymes and cytokines as well as their scavenging function. They are also links to adaptive immunity through presentation of antigens to T cells via MHC molecules and by cytokines promoting adaptive immune reponses. Macrophages are strategically placed in the body at places where microbes may enter and are rapidly activated upon encounter with microbes. Binding of microbes to the PRRs leads to ingestion of the microbes and to activation of the phagocyte. The activated macrophages secrete cytokines that leads to recruitment of more macrophages and neutrophils to the site of infection. The ingested microbes ends up in intracellular vesicles where they are destroyed by reactive oxygen/nitrogen species and proteolytic enzymes produced by the activated phagocyte.

1.1.1.2 Natural killer cells

Natural killer cells (NK cells) are an important component of the innate immune system through killing of virus-infected cells and cytokine production. The cytotoxic effect of NK cells is mediated by release of perforin, which makes pores in the membrane of the target cell, and of granzymes, which enter through the pores and induce apoptosis of the target cell. NK cells also respond to cytokines produced by activated macrophages by secreteting other cytokines, helping the macrophage to kill phagocytosed microbes.

1.1.1.3 Complement

The complement system consists of plasma proteins that promote inflammation and destruction of microbes. Some complement factors act as chemoattractants for inflammatory cells while others bind to the surface of microbes and promote phagocytosis by macrophages. In the late phase of complement activation, a complex of complement factors is formed that creates a membrane pore causing lysis of the microbe.

1.1.1.4 Opsonins

Opsonins are soluble PRRs that bind microbes and promote their phagocytosis and destruction by phagocytes. Examples are Mannose-binding lectin and C-reactive protein (CRP) which bind carbohydrates and phospholipids typically found on microbial cell membranes. Also factors from the complement cascade and antibodies produced during adaptive immune responses can act as opsonins.

1.1.2 Adaptive immunity

Adaptive immunity develops as a response to the infection and adapts to the nature of the antigen. The components of adaptive immunity are lymphocytes (B and T lymphocytes) and their products (antibodies and cytokines). The acquired immune response is characterized by a very large repertoire of antigen receptors and a very specific response, recognizing distinct determinants within antigens (epitopes). The T cell receptor (TCR) is expressed on the surface of the T cell while the B cell receptor (BCR) is expressed either on the surface or as a secreted antibody. The genes encoding the antigen receptors are formed by DNA recombination during the development of each cell, resulting in a large repertoire of antigen receptors. The lymphocyte specificity is extremely diverse and it is estimated that alltogether, the lymphocyte clones within one individual can discriminate 10^7-10^9 different epitopes.

The adaptive immune response is initiated when antigens accumulate in the peripheral lymphoid tissues (the spleen and the lymph nodes). Naïve antigen-specific lymphocytes that have never encountered antigens before, migrate through the lymphoid organs and are activated when they recognize an antigen. Lymphocyte activation requires two signals. The first signal is the recognition of the antigen itself by the antigen receptor on the lymphocyte. The second signal is a confirmation that the antigen really is non-self, and comes from either a component of the innate immune response or a product released by the pathogen. The antigen-stimulated cells expand and develop into effector and memory cells. The effector cells circulate via the blood to peripheral tissues where they fight the infection. The memory cells reside in the lymph nodes, peripheral tissues or circulate in the blood, waiting for the next infection. If the host is infected again, the memory cells ensure a rapid and strong response. After elimination of the infection, the adaptive immune system returns to its basal state. The adaptive immunity can be divided into two types of responses; cellular or humoral, which eliminates different types of infections.

1.1.2.1 Cellular immunity and antigen presentation

The cell mediated immune response is mediated by T lymphocytes (T cells), which recognize antigens presented on the surface of other cells. The cell mediated immune response serves to destroy intracellular microbes and kill infected cells as well as tumor cells. Effector T lymphocytes recognize antigens in peripheral tissues and consist of several functionally distinct populations. T helper cells (Th cells) are $CD4^+$ T cells that secrete cytokines which stimulate other cells in the immune response.

They activate macrophages to kill phagocytosed microbes and help B cells to differentiate into antibody-producing cells. $CD4^+$ T cells can be subdivided into helper T cell (Th) subpopulations (Th1, Th2, Th3, Th17) due to their different cytokine patterns that are particularly distinct in mice. This division is less distinct in humans. Cytotoxic T cells (CTL) are $CD8^+$ T cells that kill infected cells and tumor cells. The effector molecules of $CD8^+$ T cells are the same as those of NK cells (perforin and granzymes), which induce apoptosis of the target cell. Natural killer T cells (NKT cells) express the Natural Killer surface antigen CD161 in humans and NK1.1 in mice and respond specifically to lipid antigens. Another type of T cell, the regulatory T cell (Treg), serves to inhibit immune responses.

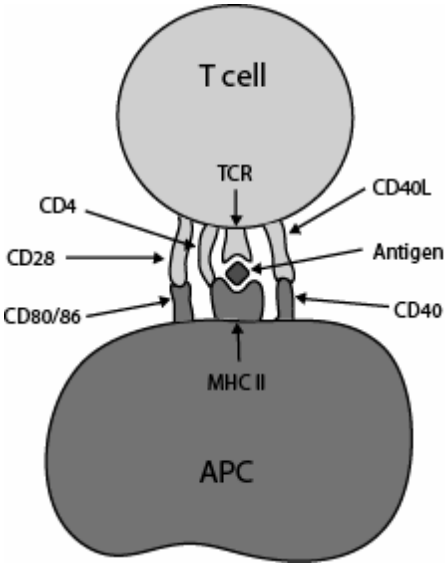

Figure 2. Interactions between T cell and antigen-presenting cell.

T lymphocytes themselves cannot bind to free antigens. The initiation and development of adaptive immune responses require binding of the TCR to small peptides derived from antigens, displayed in complex with the major histocompatibility complex molecule (MHC) on the surface of another cell. Class I MHC molecules present peptides from cytosolic proteins to $CD8^+$ CTL, enabling CTL to kill infected cells. MHC class II molecules are expressed on specialized antigen-presenting cells (APC) and present peptides derived from extracellular microbes that have been ingested by the APC (figure 2). This presentation activates Th cells, and they in turn stimulate the APC to destroy the microbe and stimulate B

cells to produce antibodies against the microbe. Another antigen presenting molecule is CD1, a MHC-like molecule that presents lipid antigens to NKT cells. The expression of MHC molecules is increased by inflammatory cytokines. In this way, innate immunity cytokines stimulate the adaptive response, and adaptive immunity cytokines amplify the adaptive response.

T cells express accessory molecules which regulate T cell activation and adhesion (figure 2). These accessory molecules make it possible for the T cell to receive co-stimulation from the APC and to bind to the extracellular matrix and to ligands on other cells. Examples of accessory molecules are CD4 and CD8, which are called co-receptors of the TCR. They bind non-variable regions of MHC II and MHC I respectively and co-signal with the TCR to induce T cell activation. Another important accessory molecule is CD28, which binds the costimulatory molecules CD80 and CD86 (B7.1 and B7.2) on APC and mediates the second signal necessary for T cell activation. Yet another effector molecule produced by activated Th cells is CD40 ligand (CD40L), binding CD40 on a number of cell types, resulting in activation of these cells.

APC are specialized in capturing antigens through for example phagocytosis, displaying them to lymphocytes and providing the second activating signal via costimulatory molecules and cytokines, stimulating T cell activation, proliferation and differentiation. Several cell types can function as APC in different situations. Macrophages present antigens to effector T cells during cellular immune responses and B cells function as APC for T helper cells during humoral immune responses, but the most specialized APC are dendritic cells (DC). DC are the most effective APC for initiating adaptive T cell responses. Immature DC are found in most tissues where they capture, endocytose and process foreign antigens into peptides that can be presented in complex with MHC. Microbial antigens (or adjuvant in the case of immunization) stimulate cells in the innate immune system to secrete inflammatory cytokines, which together with the microbial antigen/adjuvant activates the DC. The activated DC start to migrate with the antigen via lymphatic vessels to the draining lymph nodes. During the travel to the lymph nodes, the DC mature from a cell capturing antigen into a cell efficient in antigen presentation and T cell stimulation (figure 3). Mature DC express high levels of MHC, costimulatory molecules and cytokines for T cell activation and differentiation. DC present peptides from extracellular antigens in complex with MHC class II to $CD4^+$ T cells, but through a

process called cross-presentation, DC can also induce $CD8^+$ immunity. This happens when DC ingest infected cells or tumor cells and present antigens from them in association with MHC class I molecules, or when antigens are translocated within the DC from the MHC II pathway to the MHC I pathway. CD11c is widely expressed on DC and is used as marker of this celltype.

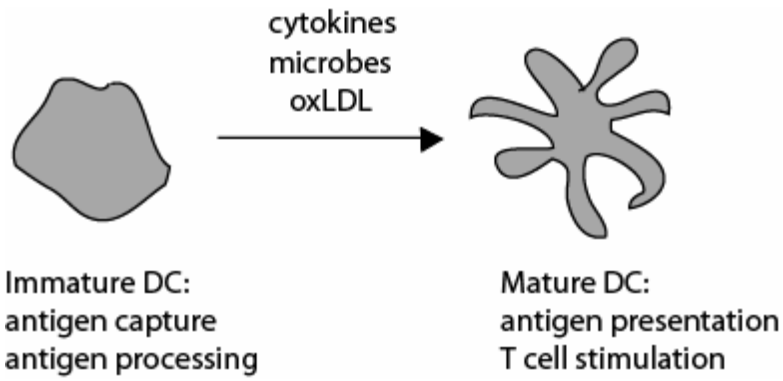

Figure 3. Dendritic cell maturation

1.1.2.2 Humoral immunity

The humoral immune response is mediated by antibodies produced by activated B lymphocytes (B cells) and recognizes free antigens. The humoral immune response serves to neutralize infectivity of the microbes and target them for elimination by other cells in the immune system. Antibodies can bind almost any kind of extracellular macromolecule. The specificity of the antibody depends on the N-terminal Fab domains, whereas the C-terminal Fc chain determines the effects. Naïve B cells express membrane bound antibodies, but when B cells differentiate into effector cells they switch to production of secreted antibodies. Activation of B cells occurs in peripheral lymphoid organs, and only the antibodies circulate and mediate their effect whenever they encounter antigen. Some antibody-producing cells change into plasma cells, which are effective antibody producers. They migrate to the bone marrow where they produce antibodies for a long time, providing immediate protection upon re-infection.

The first signal for activation of B cells is binding of antigen to the BCR. The second signal necessary for B cell activation comes from the complement cascade. Antigens bound by the BCR are internalized, and if they are proteins they are

processed and presented via the MHC class II pathway. Antigen binding also induces expression of the B7 costimulatory molecules on the B cell. Th cells can then recognize the antigen presented by the B cell and bind to B7, leading to expression of CD40L on the T cell and secretion of cytokines. In response to CD40L and cytokines, the B cells will undergo isotype switching meaning that the B cell will switch from producing membrane antibodies of IgM and IgD isotype into one of the other secreted isotypes; IgG, IgA or IgE. Different cytokines regulate switching into different isotypes. The Th2 cytokine IL-4 is the switch factor for IgE and IgG1, while the Th1 cytokine IFNγ is inducing IgG2a, and TGFβ in mucosal tissues leads to IgA production. In this way, Th cells stimulate B cell clonal expansion, isotype switching and differentiation into memory B cells. Some antigens, such as certain polysaccharides and lipids, can stimulate B cell responses without help of T cells. These antigens are called Thymus independent antigens and the antibodies induced are mainly low affinity IgM.

The binding of antibodies to antigens trigger several different effector mechanisms that eliminate the antigen. Antibodies neutralize microbes and toxins by sterical hindrance of their interaction with cells. Antibody-coated (opsonized) particles are recognized by phagocytes leading to ingestion and destruction of the particle. Antibodies bound to cells are recognized by NK cells resulting in antibody-dependent cell-mediated cytotoxicity. Antibodies also activate the complement cascade leading to opsonization, phagocytosis, inflammation, enhancement of humoral responses and lysis of the microbe. The antibody isotype determines its functions. IgM activates complement while IgG additionally act in opsonization and antibody-dependent cell-mediated cytotoxicity. IgA is the antibody in mucosal immunity and IgE mediates allergic responses and killing of parasites.

1.1.3 Cytokines

Cytokines are secreted by cells of the adaptive and innate immune system and act as growth and differentiation factors for many cell types. Cytokines are produced in response to antigens and bind to specific membrane receptors on target cells, activating new functions of these cells. In this way, cytokines regulate immune and inflammatory reactions. Some cytokines, for example TNFα, IL-1 and IL-12 are produced by phagocytic cells in response to infection and act as mediators and regulators in innate immunity. TNFα and IL-1 act on the endothelium and induce adhesion molecules promoting attachment of leukocytes. IL-12 stimulates T cells and

NK cells to produce IFNγ, which in turn activates macrophages. Other cytokines are produced by T cells in response to antigen recognition and have functions that regulate cells in the activation and effector phase of adaptive immune responses. Different kinds of antigens will induce different kinds of Th cells. Intracellular microbes (virus and bacteria) induce Th1 cells, which secrete IFNγ and TNFα that will activate phagocytes and help B cells to produce opsonizing antibodies. Parasites on the other hand, stimulate Th2 responses and the production of IL-4, IL-5 and IL-13 promoting allergic reactions and antibody production. The Th1 cytokines oppose the actions of the Th2 cytokines and vice versa. The newly identified Th17 subset produces IL-17 and plays a central role in inflammation and autoimmunity.

Finally, chemokines constitute a large family of cytokines regulating migration of leukocytes. Their function is to recruit immune cells to sites of infection and to regulate traffic of leukocytes through peripheral tissues.

1.2 APOLIPOPROTEIN E

Apolipoprotein E (apoE) is a component of HDL and VLDL. It is best known for its role in the transport of cholesterol and other lipids between the peripheral tissues and the liver. But apoE is a multifunctional protein with effects on coagulation, oxidative stress, glial cell and neuronal cell homeostasis, adrenal function, central nervous system physiology, cell proliferation and inflammation. ApoE polymorphisms modulate the susceptibility to several diseases, particularly neurodegenerative disorders and atherosclerosis [3, 4]. The presence of the so called ε4 allele is associated with an elevated risk for coronary artery disease in humans, partly due to increased levels of LDL [5]. However, it is likely that also other roles of apoE contribute to the effect. This idea is supported by the findings that apoE is produced locally in the intima and that apoE possesses several atheroprotective properties in addition to its role in cholesterol transport in the blood, including promoting cholesterol efflux from macrophages, reducing oxidative stress and inhibiting inflammation [6-10].

Studies in mice demonstrate that very small amounts of circulating apoE is enough to rescue apoE knockout (*Apoe*$^{-/-}$) mice from atherosclerosis [11-16]. Several studies have tried to distinguish between the effect of apoE on lipoproteins and on other anti-atherogenic properties. The results demonstrate that the majority of the apoE present in the lesions are produced locally by macrophages, and that macrophage-produced apoE can protect from atherosclerosis without affecting the lipoprotein profile [17,

18]. Additionally, it has been shown that apoE expressed by non-macrophages can enter the vessel wall and participate in the protection [14].

The role of apoE in inflammation is interesting. apoE inhibits SMC migration and proliferation [21], and can suppress mitogen activated proliferation and cytokine production of both $CD4^+$ and $CD8^+$ T cells [22, 23]. With one exception [24], all studies together support the idea that apoE is atheroprotective, and that apoE can act by additional mechanisms to lowering of plasma LDL.

1.3 THE IMMUNE RESPONSE IN ATHEROSCLEROSIS

The traditional risk factors for atherosclerosis are hypercholesterolemia, smoking, male gender, hypertension, diabetes and age. However, during the least decades, it has become apparent that the immune system also plays an important role in atherogenesis [25-28]. The initial observations showing involvement of the immune system in atherosclerosis was made in patient material [29, 30]. The transgenic and knockout techniques then made it possible to create mouse models of the disease and to study the roles of specific components of the immune system in atherogenesis. Atherosclerotic mouse models have therefore contributed to the growing knowledge about immune mechanisms in this disease. The most commonly used models for atherosclerosis are the Apo E knockout (*Apoe*$^{-/-}$) mouse and the low-density lipoprotein receptor knockout (*Ldlr*$^{-/-}$) mouse. A large number of studies in these models demonstrate that modulation of adaptive as well as innate immune components affects both the rate of atherogenesis and the composition of the lesions.

1.3.1 Pathogenesis of atherosclerosis

The most important risk factor for atherosclerosis is excess of the cholesterol-rich particle low-density lipoprotein (LDL). When the plasma level of LDL is increased, LDL particles will penetrate into and accumulate in the arterial wall where it is trapped by binding to proteoglycans (figure 4) [31, 32]. In the arterial wall, LDL is modified by oxidative and enzymatic processes [33-35]. Oxidized LDL (oxLDL) is a potent pro-inflammatory agent. It acts on the cells in the intima and induces expression of inflammatory cytokines, chemokines and adhesion molecules causing monocytes and lymphocytes to adhere to the endothelium and migrate from the blood between the endothelial cells into the intima, and promotes differentiation of monocytes into macrophages (figure 4) [34, 36-39]. The adhesion molecule VCAM-1 is especially interesting in atherosclerosis since it binds particularly those classes of leukocytes found in early atheromas; monocytes and T cells. VCAM-1 expression

precedes leukocyte infiltration in animal models of atherosclerosis through mechanisms depending on inflammatory signals started by the modified lipoproteins accumulating in the arterial wall [40-42]. Mice with blocked function of VCAM-1 or its counter-receptor VLA-4 exhibit reduced atherosclerosis development [43, 44].

The arterial macrophages will engulf and degrade the modified LDL in the intima. When the load of cholesterol derived from oxLDL cannot be mobilized from the macrophage, cholesterol accumulates in droplets in the cytosol and transforms the macrophages into foam cells, the characteristic cell types in the fatty streak [45]. Epitopes from the modified lipoprotein is also presented to specific T cells in the plaque [46]. Modified LDL is not tolerated by the immune system but is rather recognized as non-self and will therefore elicit an immune response and enhance the inflammation.

In response to the growth factors produced in the lesions, smooth muscle cells (SMC) from the media will migrate to the intima, start to proliferate and produce extracellular matrix to form a cap surrounding the foam cells and the free cholesterol [47]. This fibrofatty streak is the intermediate lesion of atherosclerosis. The lesion can grow for many years, with a silent inflammation inside. The advanced plaque has a center of foam cells, dead cells, and extracellular lipids surrounded by a fibrous cap consisting of smooth muscle cells and collagen-rich extracellular matrix (figure 1). Activated T cells, NKT cells, macrophages and mast cells are found mainly in the shoulder regions of the plaque where it is most prone to rupture [30, 48-52]. As the disease progresses, inflammatory cytokines inhibit SMC proliferation and collagen production and induce matrix metalloproteases (MMPs) degrading the collagen, thereby making the lesion weak and susceptible for rupture [53-56].

When the plaque ruptures, the endothelial continuity over the plaque is interrupted and exposes the plaque to the circulating blood [57]. This causes platelets to adhere and to form a thrombus (figure 1). If the thrombus occludes the vessel, blood flow is obstructed causing myocardial infarction or stroke. However, plaque rupture and thrombosis do not necessarily lead to clinical symptoms. If the blood clot does not occlude the vessel totally, a healing response follows including resorption of the thrombus. The platelets in the thrombus will stimulate SMC proliferation and synthesis of collagen. The lesion will grow and become more fibrous. As the lesion progresses, calcification may occur, giving rise to the advanced calcified plaque [58].

From the knowledge gathered about the pathogenesis of atherosclerosis, it is clear that inflammation is crucial in all stages of atherogenesis, from initiation to clinical complications.

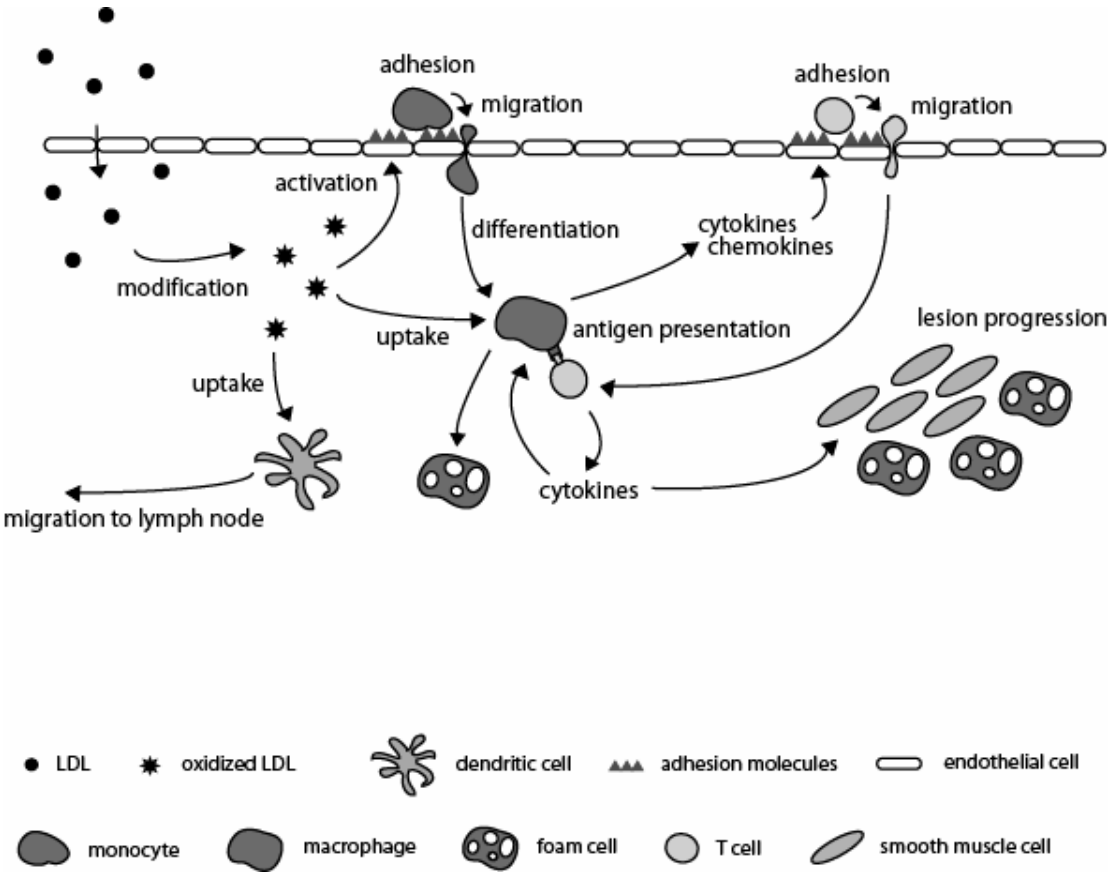

Figure 4. Atherogenesis.

1.3.2 Candidate antigens in atherosclerosis

There are several candidate antigens in atherosclerosis, but the precise epitopes for the adaptive immune response in this disease have not been entirely identified. Probably, there are not one, but many antigens which can drive the progression of atherosclerosis in different individuals under different circumstances. Several T cell-dependent and –independent antigens are present in the lesion and induce immune responses, possibly contributing to disease. One way of studying the specificity of the adaptive immune response in atherosclerosis is to measure the specificity of circulating antibodies, another to clone T cells from tissues and test their specificity.

Yet another approach is to study the effect of transferring lymphocytes from immunized donors to naïve recipients.

1.3.2.1 Oxidized LDL

The antigen that has gained most attention is oxidized LDL (oxLDL), which is thought to elicit autoimmune responses by being a modified self-protein [59]. The LDL particle consists of the protein apolipoprotein B (ApoB) together with triglycerides, cholesterol and phospholipids. Lipid peroxidation results in highly reactive products, such as malondialdehyde (MDA), which bind to free amino groups of lysine and histidine residues in ApoB as well as to phopholipids, creating immunogenic epitopes [33-35]. MDA-adducts are the major modifications found in oxLDL *in vivo* [60]. There is convincing evidence for an immune response to oxLDL in atherosclerosis. Antibodies binding to oxLDL and MDA-modified peptides from ApoB as well as to oxidized phospholipids have been detected in atherosclerotic mice and patients [60-65], and T cells cloned from human plaques respond to oxLDL in an MHC class II dependent manner [46].

1.3.2.2 Other antigens

Microbes could be another initiator of atherogenic immune responses. Evidence for the presence of microbes in the lesion exists, but their importance for atherogenesis is still controversial. Chlamydia, Herpes simplex and Cytomegalovirus have all been detected in human lesions [66] and atherosclerotic patients have antibodies to several microbes [67-71]. Of special interest are antibodies to microbial heat-shock protein 65 (HSP65). These antibodies can cross-react with human HSP60 expressed in atherosclerotic lesions, suggesting a possibility for microbial-induced autoimmunity due to the molecular mimicry between molecules on the pathogens and on self-proteins [72]. Plaque derived T cells have been shown to react to microbes and HSP65 [73-75], parenteral immunization of animals with HSP65 increases atherosclerosis [76-78] and transfer of lymphocytes from HSP65-immunized mice accelerates fatty streak formation in the recipient mice [79]. Interestingly, mucosal administration of HSP65 in mice leads to suppressed antibodies and T cell responses to HSP65 and significant reduced atherosclerosis in mice [80, 81]. This method of oral immunization has previously been demonstrated to activate the mucosa-associated lymphoid tissue and induce tolerance, ameliorating experimental autoimmune diseases such as diabetes and encephalomyelitis [82, 83].

β2-glycoprotein I (β2GPI) is a phospholipid-binding protein expressed in platelets, endothelial cells (EC) and atherosclerotic lesions [84]. Immunization of *Ldlr*$^{-/-}$ mice with this protein accelerates atherosclerosis [85], possibly through antibody-dependent activation of EC and increased uptake of oxLDL by macrophages [86]. Transfer of β2GPI-reactive lymphocytes from β2GPI immunized mice into *Ldlr*$^{-/-}$ mice accelerates atherosclerosis, and inducing oral tolerance to β2GPI suppresses atherosclerosis, illustrating the disease-aggravating properties of lymphocytes specific for this antigen [87, 88].

1.3.3 The innate immune response in atherosclerosis

The relation between innate immunity and atherosclerosis is illustrated in many studies. Cells and soluble components of the innate immune system are present in atherosclerotic lesions and inhibition of certain innate immune signaling pathways reduces atherogenesis.

1.3.3.1 Monocytes/macrophages

The recruitment of monocytes and their differentiation into macrophages and foam cells is essential for lesion formation. M-CSF is a growth factor inducing differentiation of monocytes to macrophages and mice that are deficient in M-CSF show markedly reduced macrophage accumulation in tissues and consequently almost no atherosclerosis [89-91]. As affector cells in innate immunity and inducers of adaptive immunity, macrophages contribute to atherogenesis in many ways. Thus, they promote lipid accumulation and inflammation and tend to increase the vulnerability of the plaque (Figure 5).

Activation of macrophages takes place after stimulation of their PRRs by a variety of ligands including oxLDL. Macrophages express many different ScRs, however, the importance in atherosclerosis is known only for a few of them. Genetic disruption of SR-A or CD36 has been demonstrated to reduce lesion formation in mice [92-94], probably by reducing uptake of oxLDL and foam cell formation.

The best known TLR in the context of atherosclerosis is TLR4. TLR4 is expressed in atherosclerotic lesions where it may participate in inflammatory signaling [95, 96]. Interference with TLR4 signaling in mice reduces atherosclerosis, plaque inflammation and circulating inflammatory proteins [97, 98]. In humans, attenuated TLR4 signaling is associated with decreased atherosclerosis but with higher risk of myocardial infarcion [99, 100], suggesting that TLR4-signaling plays different roles in different phases of the disease. CD14 is a non-transmembrane receptor for LPS that

initiates inflammatory responses through interaction with TLRs. A polymorphism in the CD14 promotor, resulting in increased CD14 density on monocytes, is a risk factor for myocardial infarction [101], thus demonstrating a pro-atherogenic role for innate immune responses in atherosclerosis.

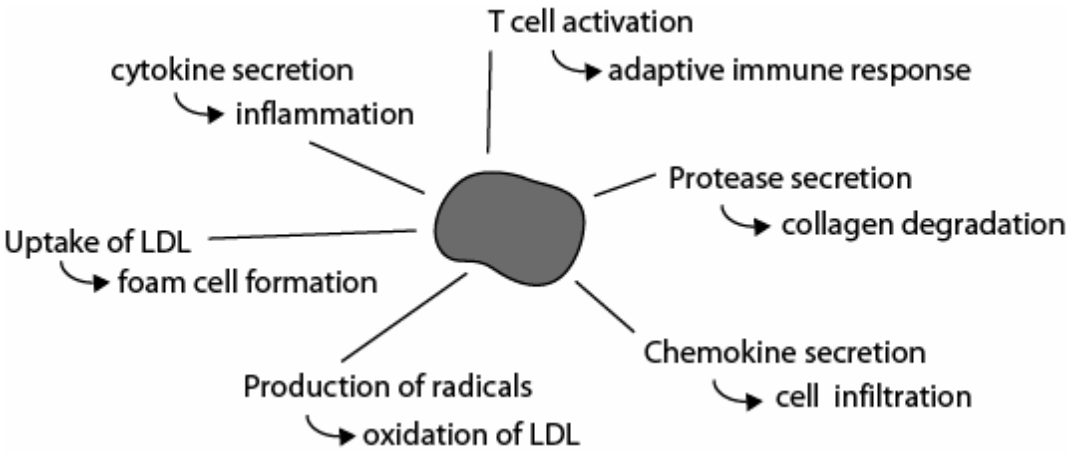

Figure 5. The role of macrophages in atherosclerosis.

1.3.3.2 Mast cells, Natural Killer cells and Neutrophils

Mast cells are present in atherosclerotic plaques at all stages of disease, accumulating in the rupture prone shoulder regions of the plaques [50, 51, 102, 103]. Mast cells also appear to be associated with vascular calcification seen in advanced plaques [103]. They are activated by binding of antigen-IgE complexes or by the complement cascade and secrete vasoactive substances, proteolytic enzymes, inflammatory cytokines and growth factors. These products influence atherogenesis by modifying lipoproteins, degrading matrix, modulating the functions of vascular and inflammatory cells, and affecting the vessel wall permeability, contractility and hemostatic properties [104]. This suggests that mast cells are not just innocent bystanders in the atherosclerotic plaque but may take part the inflammation and progression of the disease.

NK cells are present at all stages of human atherosclerotic lesion development, mostly located in the shoulder regions of the plaque, but make up a very small fraction of the total lymphocyte population [30, 105, 106]. In mice, NK cells are only found in early lesions [107, 108]. The most direct evidence for NK cell involvement

in atherosclerosis comes from a study using transgenic mice lacking functional NK cells. Replacing the leukocyte population in *Ldlr*$^{-/-}$ mice by bone marrow transplantation from NK cell deficient mice resulted in decreased atherosclerosis, demonstrating that NK cells can be pro-atherogenic [108].

Neutrophils are prevalent in eroded or ruptured plaques [109]. An increased level of neutrophils in the circulation has been identified as an independent risk factor for coronary heart disease [110], but may reflect inflammation rather that be causative. It is unclear whether neutrophils just accumulate at the site of tissue damage or if their secretion of proteinases and myeloperoxidases actually contribute to plaque progression and disruption.

1.3.3.3 Complement

Complement activation does not normally take place in healthy arteries, but activated complement is present in atherosclerotic lesions [111]. Deposition of the terminal membrane attack complex coincides with cholesterol accumulation in the intima of cholesterol fed rabbits and correlates with severity of human lesions [112, 113]. Several factors with the ability to activate complement are present in the atherosclerotic plaque, including immune complexes, CRP, pathogens, cell debris and cholesterol crystals [114-118]. Although the presence of complement is confirmed, the actual role of complement in experimental atherosclerosis remains controversial. However, a potential role of complement in atherosclerotic lesion development and maturation has been demonstrated in mice deficient in C3. Lesion from *C3*$^{-/-}$*/Ldlr*$^{-/-}$ mice were richer in lipids and macrophages and contained less collagen, and *C3*$^{-/-}$*/Apoe*$^{-/-}$/*Ldlr*$^{-/-}$ mice exhibited a more pro-atherogenic lipid profile and increased atherosclerosis, indicating a role for the complement pathway in lipid metabolism [119, 120]. In addition, the atheroprotective effect of infusion of polyspecific immunoglobulins (ivIg preparations) reduces atherosclerosis in recipient mice via complement dependent mechanisms [121, 122].

1.3.3.4 Natural antibodies

Natural antibodies are a class of germline encoded antibodies, mostly IgM, that are produced by self-renewing B1 type B cells and without T cell involvement. One of these natural IgM is the T15 antibody that provides protection against pneumococcal infections [123]. Some cloned IgM antibodies from *Apoe*$^{-/-}$ mice recognizing oxLDL are identical in their variable region to the T15 antibody. These cloned IgM bind to phosphorylcholine, which is present in the cell wall of pneumococci, in oxidized

phospholipids and on apoptotic cells, and inhibit uptake of oxLDL and apoptotic cells into macrophages [63, 64, 124-126]. Inhibited uptake of oxLDL might be atheroprotective since it would inhibit foam cell formation. These natural antibodies can be induced in mice by pneumococcal vaccination, which protects against atherosclerosis [127]. Also immunization of mice with MDA-LDL leads to increased titers of these natural antibodies [128]. In addition, passive immunization of mice with a monoclonal antibody against phosphorylcholine reduces atherosclerosis in vein grafts, further supporting an atheroprotective role for natural antibodies to phosphorylcholine in atherosclerosis [129].

1.3.4 The adaptive immune response in atherosclerosis

The involvement of adaptive immunity in atherosclerosis has been investigated in many studies and there are several pieces of evidence that a specific immune response occurs in atherosclerotic lesions [130]. Adaptive immunity can mount both protective and detrimental responses during the progress of atherosclerosis, and the balance determines the outcome (figure 6). All cell types of the adaptive immune response can be found in atherosclerotic lesions. T cells are abundant in the lesions and many of them are in an activated state [131]. They express adhesion molecules and other activation markers, secrete cytokines and are found to co-localize with antigen presenting cells. Antibodies and B cells are found in the lesions, although the B cells are few and are more abundant in the adventitia surrounding the vessels. The presence of dendritic cells and monocyte-derived macrophages in the lesion indicates that professional antigen presentation and immune activation takes place in the plaque. The activation of T cells and macrophages leads to a cascade of cytokines that induce an inflammatory state and modulates the functions of other cells in the plaque.

The role of adaptive immunity in atherosclerosis has been extensively studied in mouse models by crossing of *Apoe*$^{-/-}$ or *Ldlr*$^{-/-}$ mice into immunodeficient backgrounds, creating hypercholesterolemic mice lacking mature B and T cells. When *Apoe*$^{-/-}$ mice were crossed with Rag-1$^{-/-}$ or Rag-2$^{-/-}$ mice, lesions in the aortic root decreased with 60-80% compared to immunocompetent mice [132, 133]. In contrast, when the animals were fed a high fat diet to accelerate the disease, the lesion size and stage was not significantly affected by the immunodeficiency anymore [132, 134]. Atherogenesis in *Ldlr*$^{-/-}$ mice on western diet was also altered by immunodeficiency, such as in Ldlr/Rag-1 double knockout mice. Young mice had 54% decreased atherosclerosis in the aortic root and less mature plaques compared to

Ldlr$^{-/-}$ mice [135]. However, with time other atherogenic stresses obviously compensated for the absence of lymphocytes in these double knockout mice, and in older mice the differences in lesion size were not significant anymore. The crossing of *Apoe*$^{-/-}$ mice with SCID mice generated mice that had 70% less lesions than *Apoe*$^{-/-}$ mice [136]. These studies together support the thought that adaptive immunity influences the development of atherosclerosis, primarily in early stages of atherogenesis and when levels of cholesterol are moderate. However, they also conclude that the adaptive immune system is not obligatory for initiation and progression of atherosclerosis under conditions of extreme hypercholesterolemia. The results emphasize the importance of timing and precaution when interpreting data from studies of immune defects under high fat diet. In addition, a possibility remains that different subsets of lymphocytes could have different effects and both promote and antagonize atherogenesis. This would explain why in these models, in which all lymphocytes are lost, the net effect sometimes might be none at all. To unravel the role of different subsets of lymphocytes in atherogenesis, many clever experiments have been performed and several interesting studies are still ongoing.

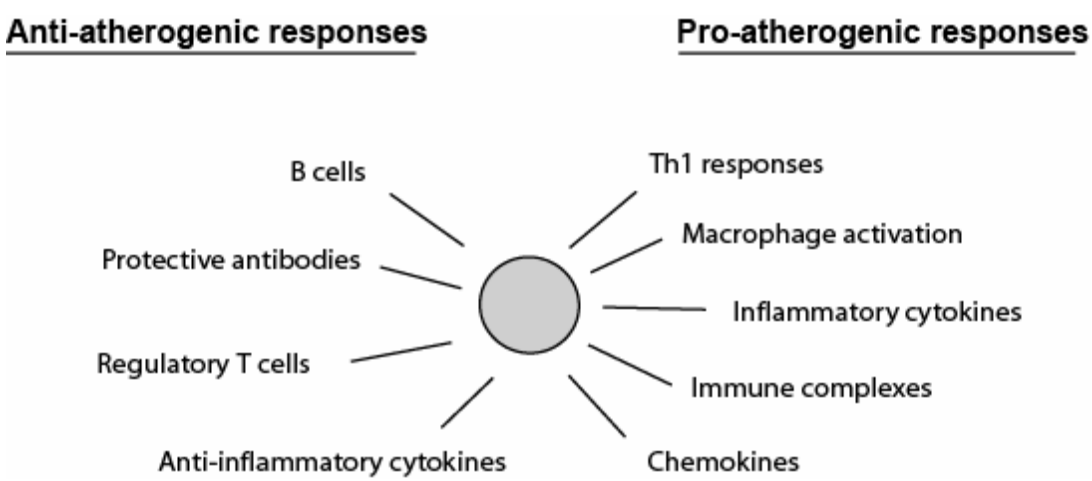

Figure 6. The role of adaptive immunity in atherosclerosis.

1.3.4.1 T cells

T cells were first described in human atherosclerotic lesions in 1985 [29]. Today, the concept that atherosclerosis is an inflammatory disease is well established and the knowledge about the complex immunological network that T cells are part of in

atherogenesis is constantly expanding. Most T cells in atherosclerotic lesions are $CD4^+$ memory or effector T cells [30, 137] and the proportion of activated T cells increase when the disease gets worse [48, 131, 138]. The activated T cells in the plaque might be a result of recruitment and/or retention of activated peripheral blood T cells, or local antigenic stimulation of resting T cells. T cells are present in lesions at all stages and are located in clusters, mainly in the shoulder regions of the lesions, closely to MHC class II expressing antigen presenting cells, indicating immune interaction [29, 30, 139-141]. Clonal expansion of T cells has been demonstrated in lesions of humans and *Apoe*$^{-/-}$ mice [142, 143]. This local expansion is not accompanied by a similar expansion in the peripheral blood, suggesting that T cell activation and expansion actually occur within the lesion.

Also in *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice, $CD4^+$ T cells are the predominant T cell subset [144, 145]. Several lines of evidence support the importance of $CD4^+$ T cells in lesion formation. Adoptive transfer of $CD4^+$ T cells from aged *Apoe*$^{-/-}$ mice to immunodeficient *Apoe*$^{-/-}$/SCID mice results in homing of T cells to the lesions and accelerated atherosclerosis to a level almost as high as in the immunocompetent mice [136]. Along this line, depleting $CD4^+$ cells by anti-CD4 antibodies or genetic disruption of the CD4 gene protects C57BL/6 mice on high cholesterol diet from fatty streak development [146]. Studies on *CD4*$^{-/-}$/*Apoe*$^{-/--}$ mice show that mice deficient in $CD4^+$ T cells still have lesions, but smaller ones, suggesting that $CD4^+$ T cells are important for progression of atherosclerosis, but are not an absolute requirement for its initiation [147, 148].

If there is a role for $CD8^+$ cytotoxic T cells in atherosclerosis is still unclear. *Apoe*$^{-/-}$/$CD8^{-/-}$ mice show no changes in lesion development compared to *Apoe*$^{-/-}$ mice [149]. However, it has been demonstrated that triggering of $CD8^+$ T cells reactive to a foreign antigen expressed by vascular SMC promotes atherosclerosis [150].

The presence of NKT cells and CD1 molecules in atherosclerotic lesions suggests that NKT cells might participate in atherogenesis [52, 151-153]. Studies in *Apoe*$^{-/-}$/*CD1*$^{-/-}$ mice and in *Ldlr*$^{-/-}$/*CD1*$^{-/-}$ mice have shown that CD1 deficient mice develop smaller lesions [152-155], further supporting a role of NKT cells in atherosclerosis. The effect of CD1 deficiency was lost in older mice, suggesting that the role of NKT cells is most important in early stages of lesion development [152-155]. Additionally, activation of NKT cells by injection of a synthetic ligand leads to increased lesion formation in mice [152-155]. Alltogether, these data support that NKT cell activation

by recognition of CD1 restricted lipid antigens might be important in the initiation and early stages of atherosclerosis.

Regulatory T cells (Treg) suppress the effector function of other immune cells, through cell-to-cell contact and secretion of the anti-inflammatory cytokines IL-10 and TGFβ. Two types of Treg have been implicated in the control of atherosclerosis-related immunity. $CD4^+CD25^+$ Treg express the transcription factor FoxP3, produce TGFβ and suppress via contact dependent mechanisms [156]. These cells are present in human plaques and in the aorta of *Apoe*$^{-/-}$ mice [157, 158]. Transfer of $CD4^+CD25^+$ Treg to *Apoe*$^{-/-}$ mice decreases atherosclerosis and injection of anti-CD25 antibodies in *Apoe*$^{-/-}$ mice inhibits the function of these cells and increases atherosclerosis, illustrating the important suppressive role of Treg in slowing down atherogenesis [159, 160]. The anti-CD25 treatment had no effect on lesion size in mice with abbrogated TGFβ signalling, illustrating that $CD4^+CD25^+$ Treg reduce atherosclerosis by producing TGFβ [159]. Another type of Treg called Tr1, suppress through secretion of IL-10 [161]. So far, one study implies that Tr1 cells can be used to inhibit atherosclerosis, through a bystander effect independently of antigen specificity [162].

1.3.4.2 B cells and antibodies

B cells are found both in early fatty-streak lesions and full-blown plaques of atherosclerotic animals and are abundant in the adventitia surrounding diseased vessels [163]. Several studies have detected both IgG and IgM in atherosclerotic plaques [30, 62, 163, 164]. The presence of immunoglobulins might reflect infiltration from the circulation and/or local synthesis by plasma cells, alternatively reduced lymphatic drainage from the lesion area. The effect of B cell deficiency in atherosclerosis has been investigated. Bone marrow transfer from B cell deficient μMT mice to *Ldlr*$^{-/-}$ mice showed that B cells and/or autoantibodies play a protective role in both early and late atherosclerosis [165]. This would suggest atheroprotection by B cell transfer, and such activity has been demonstrated. Transfer of B cells from aged atherosclerotic *Apoe*$^{-/-}$ mice into young *Apoe*$^{-/-}$ mice protected the recipients from advanced disease [166]. Transfer of B cells from young *Apoe*$^{-/-}$ mice protected to a lesser extent, implying that the protective response includes development of adaptive immunity over time.

The precise mechanism by which B cells reduce atherosclerosis is unknown, but most attention has been given to antibodies recognizing oxLDL. The hypothesis that humoral immunity can protect against atherosclerosis is supported by a large number

of experimental studies evaluating the effects of immunization on atherosclerosis, finding that immunization with oxLDL induces antibodies to oxLDL and reduces atherosclerosis [167-171]. Protective immunity seems to correlate with titers of anti-oxLDL IgG antibodies, suggesting that antibodies carry the protection achieved by immunization, and implying that antigen-specific $CD4^+$ T cells are activated and helping in the isotype-switch [121, 166, 171]. This process would involve immune recognition of peptide fragments, and indeed, antibodies to peptides of ApoB have been detected in human plasma [65]. Additionally, immunization with certain peptide sequences from ApoB resulted in reduction of atherosclerosis and plaque inflammation in *Apoe*$^{-/-}$ mice [172-174]. Treatment with recombinant human IgG against some of the protective peptides reduced plaque size in *Apoe*$^{-/-}$ mice [175]. These findings support the notion that it could be possible to develop an atheroprotective vaccine based on the ApoB derived peptides. Surprisingly, oxLDL immunization reduces atherosclerosis also in *CD4*$^{-/-}$ mice, showing that non-T cell-dependent responses are involved in the protection by immunization [147]. T cell-independent protective responses could include natural antibodies, T cell independent antibodies and innate immune mechanisms.

One can speculate that antibodies to oxLDL could protect against atherosclerosis by eliminating oxidized lipoprotein particles from the circulation or preventing their uptake by macrophages. Results from studies in *Ldlr*$^{-/-}$ and *Apoe*$^{-/-}$ mice have correlated titers of MDA-LDL antibodies with extent of atherosclerosis [62, 171, 176]. In humans however, the picture seems to be more complicated. Several clinical studies have shown the presence of circulating autoantibodies to oxLDL and to modified peptides from ApoB, both in healthy individuals and in patients with cardiovascular disease [33, 65, 177-182]. The question whether there is an association between antibodies against oxLDL and cardiovascular disease in humans and with plasma levels of oxLDL is however still debated. The conclusions from the clinical studies are mixed, finding no or an inverse correlation between antibody levels and degree of atherosclerosis or levels of oxLDL [61, 65, 178, 179, 181]. The reason for the difficulty in relating oxLDL antibodies to disease can be that oxLDL is a very complex particle containing several different epitopes, and it might be the case that some of these epitopes induce atheroprotective immune responses and some induce atherogenic responses. Additionally, different subclasses of Ig may have different effect on the disease process. B cells might also have anti-atherogenic properties

beyond antibody production. It is possible is that the B cells themselves inhibit lesion formation or stabilize the plaque by cell-to-cell contacts or by secreting cytokines, suppressing APC activity or inhibiting the Th1 pathway [183].

A possible pro-atherogenic effect of antibodies could be formation of immune complexes. The idea that circulating immune complexes involving modified LDL (LDL-IC) are atherogenic is supported by *in vitro* data showing that such immune complexes can cause macrophage activation and increased cholesterol accumulation [184]. The components of LDL-IC, oxLDL and antibodies binding oxLDL, are present in lesions, interacting with macrophages [60, 117, 185]. High concentration of plasma LDL-IC correlates with the development of coronary artery disease in diabetic patients [186]. However, the presence of these immune complexes in plasma does not prove their atherogenicity *in vivo*, although they may be a marker of disease.

Finally, even if there is a significant association between antibody levels and disease, it does not clarify whether the antibodies are part of a protective or detrimental immune response or just a marker of progressing atherosclerosis. The function of antibodies may well be context-, antigen- and isotype-dependent. Obviously, a lot more need to be clarified before we understand the role of humoral immunity in atherosclerosis.

1.3.4.3 Dendritic cells

Dendritic cells (DC) were first identified in human arteries and atherosclerotic lesions in 1995 [187, 188]. Since then, interest in the importance of vascular DC (VDC) in atherosclerosis has grown, but the pathophysiological significance of VDC as inflammatory and immune activating cells in atherosclerosis is still poorly understood.

The data supporting an involvement of DC in atherogenesis mostly rely on immunohistological findings. VDC are present in atherosclerotic lesions of humans, *Apoe*$^{-/-}$ mice and hypercholesterolemic rats [187-191]. VDC are found in areas of the healthy human aorta known to be predisposed for atherosclerosis and can be found in carotid arteries of young individuals [105, 187, 192]. The presence of DC at this stage, when no atherosclerosis is present, supports the concept that DC are involved in the early events of atherosclerosis. It has been suggested that VDC screen the arterial wall for antigens and then migrate to the local lymphoid tissue where they maintain tolerance to self antigens and elicit immune reponses to harmful antigens. When atherosclerotic lesions are evolving, DC are more abundant in the lesions than

in the healthy vessel wall. At this stage, DC are found in the intima and adventitia where they form clusters with T cells, as well as in the para-aortic lymph nodes draining the arterial wall [193]. The VDC clustering with T cells in lesions express high levels of MHC class II, ICAM-1, VCAM-1, CD1d and the activation marker CD83, suggesting a role for DC in T cell and NKT cell activation in the lesion [193-195]. In more advanced plaques, DC accumulate in clusters with T cells and NKT cells in the rupture-prone shoulder regions, suggesting a role also in plaque destabilization [52, 141, 194]. Several activating factors for DC are present in lesions, such as necrotic and apoptotic cells, modified lipoproteins and HSP [193].

The origins of DC in atherosclerotic lesions could be multiple. Some of them might be VDC that originally patrolled the healthy artery, some can infiltrate the lesion from the vasa vasorum or from neovessels, or they might differentiate from monocytes infiltrating from the blood. The finding that DC are found mainly in areas of neovascularization in the intima and within inflammatory infiltrates around vasa vasorum in the adventitia suggests that these routes are important for recruitment of DC to the lesion [194]. Whatever routs the DC choose; they at some point have to interact with the endothelial layer to enter into the lesion. Studies have shown that changes in endothelial function have a significant impact on DC maturation and recruitment to the arterial wall and may play an important part in atherosclerosis. Endothelial activation by for example oxLDL, hypoxia or TNFα increases expression of adhesion molecules, leading to DC adhesion, transmigration and accumulation within the inflamed intima [196, 197]. The increased number of DC in diseased vessels could also be mediated by reduced emigration from the lesions. Trapping of DC in the vascular wall has been demonstrated [198], and the mechanisms may include reduced migratory capacity of the DC induced by hyperlipidemia [199].

Clinical findings supporting the role of DC in atherosclerosis are few, but increasing. It was recently reported that the function of DC is increased in patients with unstable angina [200]. Inhibiting recruitment and maturation of DC in the vessel wall may have an application in reducing cardiovascular disease. Indeed, studies using statins have showed decreased DC adhesion, transmigration and maturation [141, 197, 201], suggesting that part of the antiatherosclerotic effects of statins may be caused by the inhibition of DC.

Even though a lot of data suggests that DC play a part in atherosclerosis, the association is incompletely understood and the hard evidence is still missing for a necessary role of DC in atherosclerosis.

1.3.5 Cytokines and costimulatory molecules in atherosclerosis

The cross-talk between cells in an inflammatory site, for example an atherosclerotic lesion, is mediated by soluble mediators; such as cytokines and eiconsanoids, and by surface molecules; the costimulatory molecules.

1.3.5.1 Cytokines

Cytokines are mediators of intercellular communication in the immunological network and can have pro-inflammatory or anti-inflammatory properties. Examples of pro-inflammatory cytokines are TNFα, IL-1, IL-12, IL-18 and IFNγ, and anti-inflammatory cytokines are IL-10 and TGFβ [202]. The crossbreeding of atherosclerotic mouse models with mice deficient in genes encoding cytokines or cytokine receptors has been very useful in studies of atherosclerosis, for generating knowledge about the role of different cytokines in disease development. In the atherosclerotic plaque, APC and T cells stimulate each other to production of inflammatory cytokines, which in turn increase the expression of adhesion molecules, chemokines, scavenger receptors and matrix-degrading proteases, resulting in progression of the lesion [130]. Oxidized lipids, including oxLDL, are the most likely triggering factor for cytokine production in early atherosclerosis [202]. Several cytokines have been studied extensively in relation to atherosclerosis and both pro and anti-atherogenic cytokines have been identified.

IFNγ

The pro-inflammatory Th1 cytokine IFNγ has emerged as a key player in the development of atherosclerosis. IFNγ is detected in lesions produced by lesional T cells and by other cells implicated in atheroslerosis such as NK cells, NKT cells, macrophages and smooth muscle cells [29, 46, 108, 131, 154, 203-205]. Mice deficient in this cytokine or its receptor exhibit reduced atherosclerosis and injection of the recombinant cytokine increases lesion size [206-209]. Also injections of IL-12 or IL-18, cytokines acting as IFNγ releasing factors, enhance disease in mice [210, 211]. The atherogenic properties of IFNγ are many and diverse, and involve pro-inflammatory and destabilizing mechanisms, leading to larger and more vulnerable plaques (table 1) [212, 213]. Surprisingly, bone marrow transplantation from IFNγ$^{-/-}$ mice to *Ldlr*$^{-/-}$ mice resulted in increased atherosclerosis, suggesting that under certain

conditions, IFNγ can have anti-atherogenic effects [214]. These effects might involve decreased uptake of oxLDL in macrophages via downregulation of LDL-related protein, scavenger receptor A and CD36, inhibition of lipoprotein lipase and lipoprotein oxidation and downregulation of MMP9 expression, all of which has been demonstrated *in vitro* [212, 213].

Table 1. Pro-atherogenic effects of IFNγ

Th1 response ↑
MHC class II expression ↑
Costimularoty molecule expression ↑
Adhesion molecule expression ↑
Protease secretion ↑
Chemokine secretion ↑
Inflammatory cytokine secretion ↑
Reactive radical release ↑
Macrophage cholesterol efflux ↓
SMC proliferation ↓
Collagen synthesis ↓

IL-12

IL-12 is mainly produced by monocytes, macrophages and dendritic cells and acts by inducing antigen presenting and costimulatory molecules on APC and by inducing Th1 development. IL-12 is abundant in lesions of humans and *Apoe*$^{-/-}$ mice [210, 215]. *In vivo* administration of IL-12 increases IFNγ expression in the aorta and aggravates atherosclerosis in *Apoe*$^{-/-}$ mice [210]. Additionally, *Apoe*$^{-/-}$ mice deficient in IL-12p40 display reduced atherosclerosis [216]. However, these mice also lack IL-23, since the IL-12p40 subunit is shared by both cytokines. Therefore IL-23 deficiency might contribute to the phenotype of the IL-12p40$^{-/-}$ mice.

IL-18

IL-18 is produced by monocytes/macrophages, dendritic cells and several non-hematopoetic cell types. A signaling chain of IL-18, IL-12 and IFNγ acts to stimulate Th1 responses. IL-18 stimulates T cells to produce IFNγ, which in turn activates macrophages to release IL-12, which acts in synergy with IL-18 to induce IFNγ in macrophages, T cells, NK cells, dendritic cells and even in SMC [205, 217-219]. The pro-atherogenic effect of IL-18 was demonstrated in *Apoe*$^{-/-}$ mice treated with IL-18

binding protein and in *Apoe*$^{-/-}$ mice treated with recombinant IL-18 as well as in *Apoe*$^{-/-}$/*IL-18*$^{-/-}$ mice [211, 220, 221]. It was proposed from clinical studies that IL-18 is a strong predictor of cardiovascular death in stable and unstable angina [222]. The pro-atherogenic effect of IL-18 is mediated through IFNγ [211].

TNFα

TNFα is produced by Th1 cells, macrophages and other cell types, is pro-inflammatory and promote autoimmune diseases [202]. TNFα also inhibits lipoprotein lipase, leading to hypertriglyceridemia and reduced fatty acid oxidation, and stimulates production of oxygen and nitrogen radicals. Clearly, TNFα possess several pro-atherogenic properties and inhibition of TNFα by disruption of the TNFα gene reduces atherosclerosis in *Apoe*$^{-/-}$ mice [223, 224].

IL-4

IL-4 is produced by Th2 lymphocytes, eosinophils, basophils and mast cells. It promotes allergic responses and synthesis of IgE. IL-4 inhibits Th1 differentiation and is therefore protective against many Th1-mediated diseases [202]. However, the role of IL-4 in atherosclerosis seems to be complex and conducted through several pathways, acting both to promote and inhibit atherosclerosis. When bone marrow was transferred from IL-4$^{-/-}$ mice to irradiated *Ldlr*$^{-/-}$ mice, lesions were surprisingly decreased [225]. Similarly, in *Apoe*$^{-/-}$ mice deficient in the IL-4 gene, lesions were reduced [216]. However, IL-4 injections into C57Bl/6 mice decrease fatty streak formation [146]. The pro-atherogenic properties of IL-4 could be explained by IL-4 acting on cell types other than T cells. For example, IL-4 stimulation can increase lipid peroxidation, enhance adhesion and attraction of leukocytes via VCAM-1 and MCP-1 [226, 227], and increase uptake of oxLDL as well as enhance foam cell formation [228, 229]. Additionally, IL-4 might destabilize the plaque or induce aneurysms by reduced collagen synthesis and increased production of proteases [230].

IL-5

IL-5 promotes production of natural IgM antibodies cross-reacting with oxLDL and may therefore act anti-atherogenically [128]. Indeed, bone marrow transplantation from IL-5 deficient mice to *Ldlr*$^{-/-}$ mice leads to enhanced lesion development [128].

IL-6

IL-6 is regarded as a proinflammtory cytokine, but may also act anti-inflammatory by inducing soluble receptors for IL-1 and TNF, leading to reduced activity of these pro-inflammatory cytokines [202]. This might be the reason why the role of IL-6 in

atherosclerosis appears complex. IL-6 treatment in C57BL/6 mice and *Apoe*$^{-/-}$ mice enhances lesion formation [231], but IL-6 deficiency in *Apoe*$^{-/-}$ mice also leads to enhanced lesion formation [232, 233].

TGFβ

TGFβ is a potent anti-inflammatory, immunosuppressive and profibrotic cytokine. Treg produce TGFβ, but all other cells in the atherosclerotic lesion are also capable of producing this cytokine. TGFβ acts on T cells, DC, macrophages, SMC and EC, and can therefore inhibit the development as well as increase the stability of atherosclerotic lesions on many levels [202]. The profibrotic properties of TGFβ stimulate collagen synthesis and expression of MMP inhibitors, and can therefore act to stabilize the plaque. TGFβ also acts anti-inflammatory by inhibiting recruitment of leukocytes, formation of foam cells and reducing adaptive immune responses [234]. The role of TGFβ in atherogenesis has been investigated in several studies. Neutralizing antibodies and soluble receptors were first used to demonstrate the atheroprotective property of TGFβ in *Apoe*$^{-/-}$ mice [235, 236]. Selectively disrupted TGFβ signaling in T cells lead to much larger and less stable lesions in mice [237, 238]. Additionally, atherosclerosis was suppressed by TGFβ delivered by an adenoviral vector in *Ldlr*$^{-/-}$ mice [239]. Finally, clinical data demonstrate that TGFβ is decreased in patients with advanced atherosclerosis [240]. Alltoghether, these data clearly illustrate the suppressive effect of TGFβ on atherogenesis.

IL-10

IL-10 is produced by B cells, macrophages, DC and T cells. IL-10 acts on the Th1:Th2 balance by downregulating the Th1-driving cytokines IL-12 and IFNγ and inhibiting Th1 responses. It suppresses T cell effector cells and APC acitivity [241], inhibits apoptosis, reduces cytokine secretion (primarily IFNγ), reduces expression of MMPs and increases expression of MMP inhibitors. This makes IL-10 an inhibitor of plaque inflammation and a promoter of its stability. IL-10 deficiency in C57BL/6 mice on high cholesterol diet promotes atherosclerosis [242, 243], and similar results were found in IL-10 deficient *Apoe*$^{-/-}$ mice [244]. IL-10 overexpression reduces lesions in mice, further supporting the protective role of IL-10 in atherosclerosis. Similar results have been shown regardless of method: by intramuscular gene transfer, by adenoviral transfer or by bone marrow transfer from IL-10 transgenic mice [243, 245-247]. Another bone marrow transfer experiment illustrated that leukocyte–derived IL-10 is required for the protective effect in *Ldlr*$^{-/-}$ mice [248]. IL-10 and TGFβ may also act anti-atherogenically through other mechanisms, including

induction of regulatory T cells, induction of tolerogenic DC, reduction of adhesion molecules and induction of other anti-inflammatory cytokines.

Chemokines
Interference with leukocyte recruiting chemokines or their receptors inhibit atherogenesis. Both MCP-1 (produced by SMC and EC) and its receptor CCR2 (expressed on monocytes, T cells and B cells) are detected in atherosclerotic plaques of *Apoe*$^{-/-}$ mice [249-253]. Atherosclerosis prone mice deficient in MCP-1 or its receptor display reduced leukocyte recruitment and up to 80% reduced lesions in the aorta, illustrating the importance of this recruitment pathway [249-252]. Also other chemokines such as fractalkine, IL-8, RANTES and IP-10 participate in leukocyte accumulation in lesions [158, 254-257].

1.3.5.2 Costimulatory molecules

Costimulatory molecules are crucial for cell-to-cell contacts in the immunological network. T cells need costimulation from the APC during the activation phase of the immune response and they signal to other cells during the effector phase through costimulatory molecules.

CD40L
The best known costimulator in atherosclerosis is CD40L. It is expressed by T cells and binds CD40 on B cells, macrophages and DC, resulting in activation of these cells [258]. In atherosclerosis, CD40-CD40L interactions lead to increased expression of chemokines, adhesion molecules, MMPs and tissue factor, contributing to recruitment of inflammatory cells and destabilization as well as increased thrombogenicity of the lesions [258]. Neutralizing CD40-CD40L interactions in atherosclerosis-prone mice, either by genetic disruption of the gene or by injection of neutralizing antibodies, reduces lesion formation, inhibits evolution of established plaques and induces a stable phenotype [259-262]. CD40 and CD40L are also expressed by non-immune cells such as EC, platelets and SMC in the atherosclerotic lesion, resulting in many possibilities for interactions. Interestingly, it was recently demonstrated that CD40L deficiency on immune cells did not affect atherosclerosis, suggesting that other non-immune cell types are responsible for the atherogenic effect of CD40L [263, 264].

OX40L
T cells express Ox40 that binds Ox40L on a variety of cells. This interaction is important for development and survival of memory T cells and is involved in

autoimmune diseases [265, 266]. Ox40L deficient mice display reduced atherosclerosis while overexpression of Ox40L increases lesion formation [267]. A polymorphism in the Ox40L gene was identified as a genetic risk factor for myocardial infarction in humans [267].

CD28

CD28 is expressed by T cells and binds CD80 and CD86 (B7.1 and B7.2) on APC. Whether these molecules contribute to atherogenic signaling is still unclear, because of contradicting results in animal studies. *Ldlr*$^{-/-}$ mice deficient in CD80/CD86 from birth displayed reduced atherosclerosis [268], while irradiated *Ldlr*$^{-/-}$ mice reconstituted with CD80/CD86 deficient bone marrow exhibited increased lesions [159]. These opposite results do not have an obvious explanation, but may depend on the presence of CD80/86 in nonhematopoietic cells.

1.3.6 Applications in atherosclerotic patients

Today we understand that the immune system plays a complex and important role in atherosclerosis. Gaining more knowledge about the atherosclerotic inflammation serves two important purposes; to find means of identifying patients at risk and to find new therapeutic targets.

1.3.6.1 Immunologic markers of atherosclerosis

Attempts have been made to correlate circulating inflammatory markers to the risk for future cardiovascular events. Indeed, plasma levels of IL-6, soluble VCAM-1, soluble ICAM-1, soluble CD40L and CRP do fulfil this correlation [269-274]. The correlations between inflammatory biomarkers and severity of atherosclerosis strongly support that inflammatory immune activation plays a role in acute coronary syndromes, and that inflammatory markers in the circulation can reflect the clinical outcome. However, the correlations are not specific for atherosclerosis and can reflect any ongoing inflammatory process. Increased knowledge about the immune mechanisms in atherosclerosis could be helpful for developing better diagnostic markers.

The identification of adaptive immune responses to plaque constituents has raised the question if markers of immune responses to certain antigens could help us determine the atherosclerotic disease activity and predict clinical outcomes. Studies have tried to correlate the plasma levels of antibodies to oxLDL with progression of atherosclerosis, but the results have been inconsistent. Probably, the role of these antibodies is too complex to be elucidated by the current methods. In addition, a

thorough characterization of the antigens involved in the initiation of the immune response is missing. Oxidation of LDL involves a number of modifications and many epitopes are formed which might induce different immune responses with varying effects on disease development. More detailed knowledge about the immunogenic epitopes of oxLDL, the types of antibodies induced and their effects are needed to clarify if antibodies could be developed into suitable biomarkers.

1.3.6.2 Immunomodulation in atherosclerotic patients

The development of anti-inflammatory therapies directed towards atherosclerotic biomarkers is a growing research field. Drugs directed to inhibit inflammatory mediators are under investigation and evaluation [275-277]. In addition to developing new drugs with the purpose to inhibit inflammation, researchers have found that established drugs exhibit anti-inflammatory properties. For example, statins are used for treatment of hypercholesterolemia and prevention of atherosclerosis. In addition to their cholesterol lowering effect, statins have been found to modulate inflammation and plaque stability through several mechanisms, involving inhibited recruitment and activation of immune cells, as well as reduced matrix degradation and thrombogenicity [278, 279]. Also thiazolidinediones (TZDs) used for glucose control in diabetes, as well as inhibitors of angiotensin-converting enzyme (ACE) and angiotensin receptor blockers used for hypertension, are now known to reduce CRP, IL-6 and vascular inflammation [280, 281]. So far, oxLDL specific immunomodulation have not been performed in patients with atherosclerotic disease, but potential treatments are under development.

2 AIMS

The studies included in this thesis aim to shed light on key components of adaptive immune reponses in atherosclerosis. More specifically, they aim to:

- clarify the role of apolipoprotein E as a modulator of immune responses.
- investigate the effect of Interleukin-18 on atherosclerosis in the absence of adaptive immunity.
- determine whether the pro-atherogenic effect of $CD4^+$ T cell transfer is antigen specific.
- explore the role of dendritic cells presenting modified LDL in the development of atherosclerosis.
- study immunization-induced atheroprotection in the absence of B cells and antibodies.

3 METHODOLOGICAL CONSIDERATIONS

3.1 MURINE MODELS OF ATHEROSCLEROSIS

Our knowledge about the pathogenesis of atherosclerosis comes from a combination of research in animal models and cell cultures, analysis of human lesions, clinical investigations in patients as well as epidemiological studies.

Figure 7. Mouse

Animal models are needed to study both early and late phases of atherosclerosis, to dissect the pathogenic mechanisms and to determine causality. Two knockout mouse strains that develop hypercholesterolemia and atherosclerosis are commonly used nowadays for studies in atherosclerosis [282]. The apolipoprotein E knockout mouse (*Apoe*$^{-/-}$) spontaneously develops hypercholesterolemia and atherosclerosis and the LDL receptor knockout mouse (*Ldlr*$^{-/-}$) does the same when fed a high cholesterol diet. Important information about the role of the immune system in atherogenesis has been provided by crossing these atherosclerosis prone mice with mice carrying targeted deletions in genes of the immune system or by performing bone marrow transplantations from immune-knockout mice.

3.1.1 ApoE knockout mice (paper I, II, III, IV, V)

ApoE is an apolipoprotein that acts as a ligand for the LDL receptor and the chylomicron remnant receptor. Deficiency in apoE therefore leads to defective removal of chylomicron remnants and intermediate density lipoprotein particles from the circulation, resulting in hypercholesterolemia. *Apoe*$^{-/-}$ mice develop atherosclerotic lesions that are similar to human plaques, progressing from fatty streaks to fibrofatty plaques and advanced lesions [283-285]. An advantage of the *Apoe*$^{-/-}$ mouse compared to other mouse models of atherosclerosis is that this mouse develops atherosclerosis spontaneously without the need of a high fat diet [282, 285]. A disadvantage is that the lipoprotein profile is dominated by elevated VLDL, which is different from the human situation where LDL usually dominates. The *Apoe*$^{-/-}$ mice used in our experiments were on a C57BL/6 background and fed standard chow.

3.1.1.1 ApoE knockout mice lacking adaptive immunity (paperII, III)

T and B cell deficient *Apoe*$^{-/-}$ mice were previously generated in our laboratory by crossing SCID mice with *Apoe*$^{-/-}$ mice [136]. SCID mice fail to develop mature T and B cells due to abnormal recombination of the TCR and immunoglobulin genes as a result of a recessive mutation in the gene coding for a DNA repair enzyme called DNA dependent protein kinase [286, 287]. Mice homozygous for this mutation have severely impaired lymphopoiesis, resulting in few if any T and B cells. Consequently, SCID/*Apoe*$^{-/-}$ mice are deficient for antibodies and immune functions mediated by T and B lymphocytes. Leakage of functional T and B cells has been reported in SCID mice [288], especially when they are over one year old [289]. All SCID/*Apoe*$^{-/-}$ mice included in our study were younger than 5 months and had neither T cells, B cells, nor antibodies.

3.1.1.2 ApoE knockout mice lacking humoral immunity (paper V)

B cell deficient *Apoe*$^{-/-}$ mice were provided by Drs Francis Bayard and Rima Elhage in Toulouse, France. These mice had been generated by crossing µMT mice with *Apoe*$^{-/-}$ mice. µMT mice are knockouts for the gene encoding the µ-chain of the B cell receptor and therefore their B cell development is arrested already at the stage of pre-B-cell maturation [290]. All µMT/*Apoe*$^{-/-}$ mice included in our study were totally deficient in mature B cells and antibodies.

3.2 EXPERIMENTAL METHODS

3.2.1 LDL isolation and modification (paper III, IV, V)

Murine MDA-modified LDL was used for immunizations, DC pulsing and *in vitro* assays. Murine LDL was used to reduce the risk of inter-species reactions and to mimic the antigen in the lesions as precisely as possible. MDA-modification was chosen since it results in adducts that are known to be present in oxLDL *in vivo* [33, 291]. Murine LDL was isolated from *Apoe*$^{-/-}$ mouse plasma by ultracentrifugation through a discontinous NaCl gradient for 20 hours and collection of the fraction between 1,020 mg/ml and 1,063 mg/ml [60]. This density cutoff contains mainly LDL particles. MDA was produced by acid hydrolysis of malondialdehyde-bis-dimethylacetal, and LDL was incubated with 0.5 M MDA for 3 hours at 37ºC to generate MDA-LDL. Unbound MDA was removed by running the sample over a PD10 buffer exchange column in order to avoid any potential side-effects induced by free MDA.

3.2.2 Immunizations (paper III, V)

For immunizations, antigen was emulsified in Freund's adjuvant at a volume ratio of 1:1. At each injection time, each mouse was immunized with 100 μg antigen. Since Freund's adjuvant itself has been shown to have effects on atherogenesis [127, 147, 292], control groups were set including untreated mice, mice immunized with PBS and mice immunized with the disease-unrelated antigen Keyhole limpet hemocyanin (KLH). In the antigen specific T cell-transfer experiment in paper III, mice were immunized exclusively with incomplete Freund's adjuvant (IFA) in order to avoid induction of T cells responsive to heat shock proteins known to be present in complete Freund's adjuvant (CFA). In the long-term experiment of paper V, the same protocol as in our previous long-term immunization studies was followed, with CFA used for the first injection and IFA for booster injections at two-week intervals [147, 171].

3.2.3 Cytokine injections (paper I, II)

To study macrophage activation induced by IFNγ *in vivo*, 100 units (10 ng) of recombinant murine IFNγ was injected intraperitoneally in each mouse. This dose corresponds to approximately 0.5 ng/g body weight. To evaluate the long term effects of exogenous IL-18 on atherosclerosis, mice received three intraperitoneal injections per week for 7 weeks. The dose given at each injection time was 1μg, which corresponds to approximately 50 ng/g body weight, resulting in a similar weekly dose as described in a previous publication [211]. To study short-term effects of IL-18, the dose was increased to 2 μg IL-18 (approximately 100 ng/g body weight), injected twice with 24 hours interval.

3.2.4 Isolation of T cells (paper I, III, IV, V)

T cells were purified in several different ways. The method of choice depended on the purpose of the isolation. The purity after the isolation was determined by flow cytometry. In paper I, T cells were enriched on home-made nylon-wool columns, resulting in 90% purity, and used for proliferation assays. In paper III and V, negative selections with antibody-coated magnetic beads were performed in order to avoid T cell activation. This method resulted in >95% pure T cells which were used for cell transfer and culture.

3.2.5 Generation and pulsing of dendritic cells (paper V)

To generate dendritic cells (DC), we cultured murine bone marrow in the presence of IL-4 and GM-CSF. Generally, this culture of bone marrow cells in GM-CSF and IL-4

leads to proliferation of myeloid cells; granulocytes, macrophages and DC [293]. Therefore, we performed purification of DC in a density gradient, resulting in >70% of the cells being highly positive for CD11c, I-A^b and costimulatory molecules. We found that the purified DC were effective in presenting antigen to T cells whereas the cells remaining after gradient purification were unable to activate T cells *in vitro*. For pulsing experiments, the purified DC were incubated at a high density with high concentration of either MDA-LDL or KLH overnight. The antigen concentration was titrated to give as high antigen uptake as possible without causing cell-toxicity. To avoid antigen effects on the maturation of the DC, pulsing was performed in the presence of LPS to ensure proper activation of all the cells.

3.3 METHODS OF ANALYSIS

3.3.1 Quantitation of plaque size (paper II, III, IV, V)

To evaluate atherosclerotic disease progression, lesion size was measured in the aortic root. Collection of sections from the appearance of the valves to 500 μm from the valves covers the major part of the lesion, and therefore the mean value from 5 sections at 100 μm intervals from the appearance of the valves was calculated and used for comparisons. Lesion development was measured in the aortic root rather than in the entire aorta since immunodeficent *Apoe*$^{-/-}$ mice exhibit no detectable lesions outside of the aortic root (Zhou et al, unpublished observation) and there is a strong correlation between the extent of atherosclerosis in the aortic root and in the aortic tree in *Apoe*$^{-/-}$ mice [294].

3.3.2 Analysis of T cell proliferation (paper I, III, IV, V)

To detect antigen specific T cell proliferation, total splenocytes, or purified T cells together with irradiated splenocytes as APC, were cultured in the presence of titrated amounts of antigen followed by incorporation of ^{3}H-thymidine. The results are presented either as cpm values, which are the counts per minute detected by a β-counter, or when baseline was different between groups, as a stimulation index which is calculated by subtracting the baseline cpm from the stimulated cpm and dividing by baseline cpm. To avoid background proliferation caused by the presence of small amounts of LDL in fetal calf serum, LDL proliferation assays were performed in serum-free medium containing ITS+, which contains insulin, transferrin, and selenous acid, and stimulates cell proliferation under serum-free conditions.

3.3.3 Staining for surface markers and intracellular cytokines (paper I, II, III, IV, V)

For specific detection of molecules expressed inside and on the surface of cells, flow cytometry and immunohistochemistry were used. Staining for surface molecules with labeled monoclonal antibodies was performed to analyze the presence of inflammatory markers and cellpopulations, or to analyze the purity of cells after isolation. Optimal dilutions of the antibodies were determined by titration. Controls were stained with isotype-matched control antibodies or without any primary antibody. When staining for intracellular proteins, all incubations were performed in a saponin-containing buffer to enable access of the antibodies into the cytosol. Results from flow cytometry are presented differently depending on the nature of the marker detected. Data for markers showing a discontinous expression, dividing cells into positive and negative populations, are presented as percent positive cells. Data for markers with a continuous expression are presented as mean fluorescence of the cells. Quantification of immunohistochemical stainings was performed when possible by counting the number of positive cells per mm^2, or by measuring the positively stained area as percent of the total lesion area.

3.3.4 Determination of antibodies and cytokines in sera and supernatants (paper II, III; IV, V)

Enzyme linked immunosorbent assay (ELISA) and cytometric bead array (CBA) were used for sensitive detection of specific antibodies, total antibody isotypes and cytokines in mouse sera and in cell-culture supernatants. Blood was obtained by heart puncture at the time of euthanasia. The serum was centrifuged at 14000g for 30 min before the analysis to remove chylomicrons that may interfere with the assays. Total levels of antibody isotypes were measured by sandwich ELISA and antigen-specific IgG- and IgM-antibodies were analyzed in ELISA plates coated with the antigen of interest (MDA-LDL or KLH). We tested the non-specific binding of the samples in plates coated with BSA. By subtracting the OD value from the BSA assay from the OD value of the MDA-LDL or KLH assay we derived adjusted OD values. However, the adjusted OD values did not change the results of the experiment, and therefore presentation of the data as raw OD values was chosen. Levels of cytokines in sera and supernatants were measured by commercial sandwich ELISA and CBA kits. The principle of CBA is similar to that of sandwich ELISA, but instead of using antibody-coated plates and enzyme-conjugated detection antibodies, CBA utilizes antibody-

coated plastic beads and fluorescently labelled detection antibodies, enabling analysis by flow cytometry. CBA is more sensitive than ELISA and requires less sample volume. Recombinant mouse cytokines were used as standards and the concentration in the samples is presented in pg/ml.

3.3.5 Real-time PCR (paper II, IV)

For detection of cytokine mRNA expression in lymphoid and atherosclerotic tissues, real-time PCR was used. The readout was arbitrary mRNA units obtained by comparing the threshold cycle value of the test sample with that of a standard curve prepared from murine spleen mRNA. Data is expressed as the ratio between the gene of interest and the housekeeping gene HPRT or 18S RNA, chosen because of their stable expression in the samples.

3.4 STATISTICS (PAPER I, II, III, IV, V)

Since the distribution of variables such as lesion size, antibody titers and cholesterol levels was unknown, we have exclusively used the nonparametric Mann-Whitney significance test for variables likely to have a skewed distribution. For comparing high and low expression in immunohistochemistry, we used the χ^2-test. The significance level was set at $P<0.05$.

4 RESULTS AND DISCUSSION

4.1 APOLIPOPROTEIN E MODULATES IMMUNE ACTIVATION

Apolipoprotein E (apoE) is a pleiotropic protein with several functions beyond cholesterol transport. Increasing the levels of apoE is clearly atheroprotective, due to positive effects on the lipoprotein profile, but probably also due to its anti-oxidative and anti-inflammatory properties [3]. It has been shown *in vitro* that apoE can attenuate T cell proliferation and suppress Th1 responses, but its immunological mechanism of action has remained unclear [22, 23, 295]. To investigate the immuno-supressing properties of apoE *in vivo*, we analyzed T cell responses to the exogenous antigen ovalbumin (OVA) in *Apoe*$^{-/-}$ and *Apoe*$^{+/+}$ mice. T cells were isolated from the draining lymph nodes of immunized *Apoe*$^{-/-}$ and *Apoe*$^{+/+}$ mice and were then co-cultured with macrophages isolated from either *Apoe*$^{-/-}$ or *Apoe*$^{+/+}$ mice (figure 8).

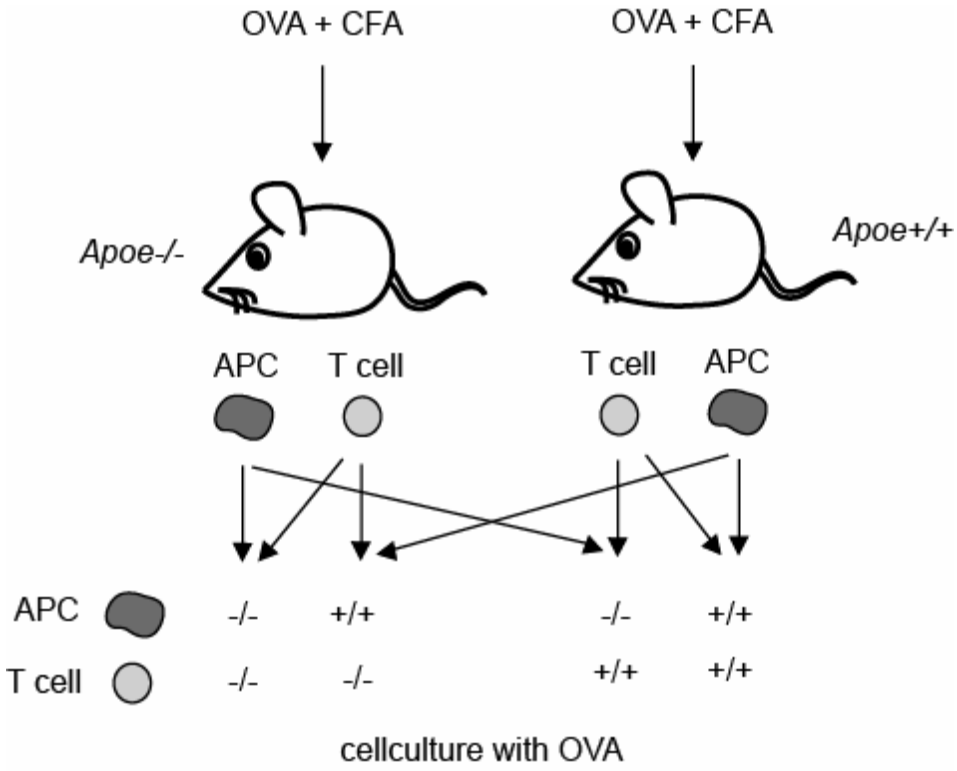

Figure 8. Experimental setup to investigate immuno-supressing properties of apoE.

We found that macrophages from *Apoe*$^{-/-}$ mice stimulated T cells from OVA immunized mice of both strains more effectively than macrophages from

Apoe$^{+/+}$mice. The same effect was observed when the cells were stimulated with the polyclonal mitogen concanavalin A (Con A) instead of OVA. Both proliferation and IFNγ secretion were enhanced in T cells activated by antigen-presenting cells from *Apoe*$^{-/-}$ mice, illustrating that the antigen-presenting function is enhanced in *Apoe*$^{-/-}$ mice.

To investigate the reason for this enhanced T cell activating property of macrophages from *Apoe*$^{-/-}$ mice, we assessed the expression of surface molecules on macrophages after *in vivo* stimulation with IFNγ. This treatment causes increased expression of the costimulatory surface proteins CD40 and CD80, and also of the MHC class II molecule I-A^{b} on macrophages. We found that these molecules were more abundandtly expressed on macrophages from *Apoe*$^{-/-}$ mice compared to *Apoe*$^{+/+}$ after IFNγ stimulation, suggesting that apoE expressed by macrophages inhibits T cell activation by reducing the density of immune stimulatory proteins on the macrophage. ApoE secreted by macrophages may therefore dampen immune activation by inhibiting the expression of MHC and costimulatory molecules. Therefore, *Apoe*$^{-/-}$ mice exhibit enhanced antigen presentation and costimulation compared to *Apoe*$^{+/+}$ mice after *in vivo* activation. When apoE is produced by the macrophage, it can be secreted or stored intracellularly, and macrophages also have receptors that can bind apoE protein [296]. The effects we find in *Apoe*$^{-/-}$ macrophages could therefore be due to lack of intracellular apoE, lack of autocrine apoE stimulation or to the fact that the macrophages were differentiated in an apoE-deficient environment. How the effects of apoE are mediated, if it is via regulation of transcription or by direct interactions with proteins or lipids inside or on the surface of the cells, is not known.

In addition to its effects on T cell activation, apoE modulates the innate immune response in mice. *Apoe*$^{-/-}$ mice are more sensitive to infections by certain bacteria, suggesting that apoE promotes the macrophage-dependent defense against microbes [297, 298]. ApoE may therefore exert the paradoxical effects of enhancing innate and dampening adaptive immune responses. Such effects could perhaps be explained by apoE-dependent control of the differentiation and/or activation of macrophages. The physiological role of this regulation is not clear, but we can speculate. In a non-inflammatory situation, apoE is produced and dampens the adaptive immune response but keeps the innate immunity ready for combating infections. However, in an inflammatory situation, IFNγ is secreted from activated T cells or cells of the innate

immune system and will inhibit apoE secretion from macrophages by increasing intracellular degradation of the newly synthesized apoE [19]. This will in turn enhance the T cell activating properties of the macrophage and the adaptive immune response. This implies an intricate, apoE-mediated regulation of immune activation.

The effects of macrophage-derived apoE are important in atherosclerosis. Reconstruction of macrophage-specific expression of apoE reduces atherosclerosis in *Apoe*$^{-/-}$ mice [12, 17], while reconstitution of C57BL/6 mice with macrophages from *Apoe*$^{-/-}$ mice increases atherosclerosis [18]. It is possible that the immunomodulatory effect by apoE on T cell activation that we have demonstrated is a contributing factor to the atheroprotective properties of apoE, and to the atheroclerosis-prone phenotype of *Apoe*$^{-/-}$ mice.

4.2 IL-18 ACCELERATES ATHEROSCLEROSIS INDEPENDENTLY OF T CELLS

IL-18 is a potent pro-atherogenic cytokine that enhances atherosclerosis in an IFNγ dependent manner in *Apoe*$^{-/-}$ mice [211, 220, 221]. T cells are known to be a major source of IFNγ, and it has been unknown if other celltypes than T cells respond to IL-18 stimulation *in vivo*. Studies *in vitro* have shown that macrophages, NK cells and even vascular SMC may secrete IFNγ upon stimulation by IL-18 [205, 217, 218]. All these cell types may therefore be important in the IL-18 response and contribute to IFNγ secretion *in vivo*. To confirm this hypothesis, we investigated the effect of IL-18 on atherosclerosis in the absence of adaptive immunity in SCID/*Apoe*$^{-/-}$ mice (figure 9).

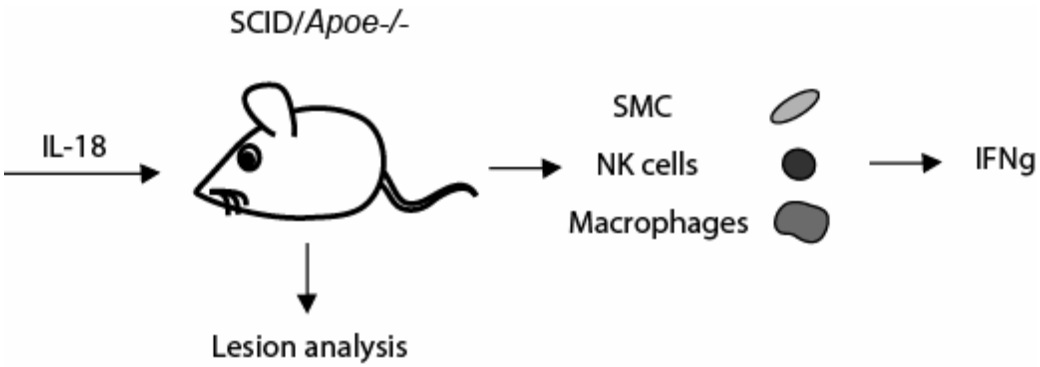

Figure 9. Experimental design to investigate the effect of IL-18 on atherosclerosis in the absence of adaptive immunity. Control animals were injected with PBS.

Our results show that *in vivo* administration of IL-18 leads to higher levels of circulating IFNγ and larger atherosclerotic lesions, despite the absence of T cells. We observed that IFNγ expression was elevated locally in the atherosclerotic lesions after systemic IL-18 treatment, together with increased levels of VCAM-1 and MHC class II, indicating increased local inflammation. Short-term treatment with IL-18 showed that IL-18 induced IFNγ expression in $CD11b^+$ macrophages, $NK1.1^+$ NK cells and the cells in the healthy vascular wall. The main cell type in the healthy vascular wall is the SMC, and they may be the source of IFNγ detected in the vessel wall. However, we can not rule out that occasional immune cells are present in the healthy vessel and contribute to the IL-18 response. WE deetcetd elevated levels of IFNγ both locally in the lesions and systemically in the blood and lymphoid orgnas after IL-18 administration. Therefore, the observed effects of IL-18 on atherosclerotic lesions could either be due to IL-18 infiltrating the lesions from the circulation and there locally inducing IFNγ and inflammation, or to secondary effects of the injected IL-18 via elevated levels of systemic IFNγ. Either way, our results confirm the pro-atherogenic effect of IL-18, and illustrate that IL-18 can influence atherosclerosis in a T cell-independent manner by inducing IFNγ production in macrophages, NK cells and vascular cells. In studies of immune mechanisms in atherosclerosis, IFNγ is most of the time referred to as a T cell cytokine promoting the disease. We can now conclude that IFNγ from non-T cells also contribute to atherogenesis.

Although IFNγ is well known as a pro-atherogenic cytokine (see table 1 in the introduction), its effects on ScRs have seemed like a paradox. IFNγ downregulates the expression of SR-A and CD36, inhibiting foam cell formation [305, 306]. Logically, this would be atheroprotective since it has been demonstrated that SR-A and CD36 knockout mice are protected from atherosclerosis [92-94]. This paradox could be explained by the finding that IFNγ upregulates expression of the ScR CXCL16/SR-PSOX, another ScR mediating oxLDL uptake and foam cell formation [300-301]. CXCL16/SR-PSOX is a ScR which is expressed in lipid-laden macrophages and SMC in human atherosclerotic lesions [299-301]. In addition, CXCL16 can be cleaved off from the cell-surface and act as a chemokine for T, NK and NKT cells [302-304]. We found increased levels of CXCL16 in the lesions after IL-18 treatment, suggesting that the pro-atherogenic role of IL-18 could partly depend on IFNγ mediated induction of CXCL16, leading to enhanced uptake of oxLDL into

macrophages leading to foam cell formation, and recruitment of NK cells contributing to the local inflammatory response.

On the other hand, ScR activity might not always be a bad thing in atherosclerosis. One can speculate that oxLDL inside foam cells would do less harm than extracellularly, since it would cause less tissue damage and the cholesterol could be eliminated from the lesion via cholesterol efflux to HDL particles. In that scenario, downregulating scavenger receptors would be pro-atherogenic, leading to accumulation of extracellular cholesterol. Indeed, it was recently demonstrated that CXCL16 deficient *Ldlr*$^{-/-}$ mice have reduced ScR activity and accelerated atherosclerosis with higher levels of apoptotic cells, MCP-1 and TNFα in the lesions [307]. This indicates a potential atheroprotective function of CXCL16 in hypercholesterolemic *Ldlr*$^{-/-}$ mice. One can speculate that the scavenger receptor functions of CXCL16 may act atheroprotective, while the chemokine functions of CXCL16 contribute to atherogenesis.

In addition to the effects of IL-18 mediated by IFNγ, it is possible that IL-18 may exert IFNγ-independent effects. For example, IL-18 can directly inhibit IL-10 production, enhance NK cell activity and increase the expression of IL-6, IL-8, IL-1, TNFα, MCP-1 and MMPs [205, 308, 309]. However, the lack of effects on atherosclerosis in IL-18 treated *Apoe*$^{-/-}$IFNγ$^{-/-}$ mice indicates that such pathways are of minor importance in atherogenesis [211].

4.3 THE ROLE OF CD4$^+$ T CELLS REACTIVE TO MODIFIED LDL

The role of CD4$^+$ T cells in atherosclerosis has been investigated in several studies [46]. T cells from lymphoid organs of *Apoe*$^{-/-}$ mice recognize modified LDL [310], transfer of CD4$^+$ T cells from aged *Apoe*$^{-/-}$ mice into SCID/*Apoe*$^{-/-}$ mice accelerates the progression of atherosclerosis and absence of CD4$^+$ cells in *Apoe*$^{-/-}$ mice leads to reduced atherosclerosis [136, 147]. To determine whether the pro-atherogenic effect of T cells is antigen specific or not, we purified CD4$^+$ T cells from MDA-LDL-immunized mice and transferred into SCID/*Apoe*$^{-/-}$ mice. CD4$^+$ T cells from mice immunized with an antigen not related to the disease, Keyhole limpet hemocyanin (KLH), and naïve CD4$^+$ T cells were used as controls (figure 10).

Our data show that mice receiving CD4$^+$ T cells from MDA-LDL immunized mice had substantially accelerated lesion progression compared with those receiving naive or KLH-primed T cells. The infiltration of CD4$^+$ T cells and the level of MHC class II expression were increased in the lesions of mice receiving MDA-LDL primed

T cells, suggesting that the MDA-LDL specific $CD4^+$ T cells homed to and expanded in the atherosclerotic lesions, where the MDA-LDL antigen accumulates. This study shows for the first time that adoptive transfer of cell-mediated immunity to oxLDL accelerates atherosclerosis.

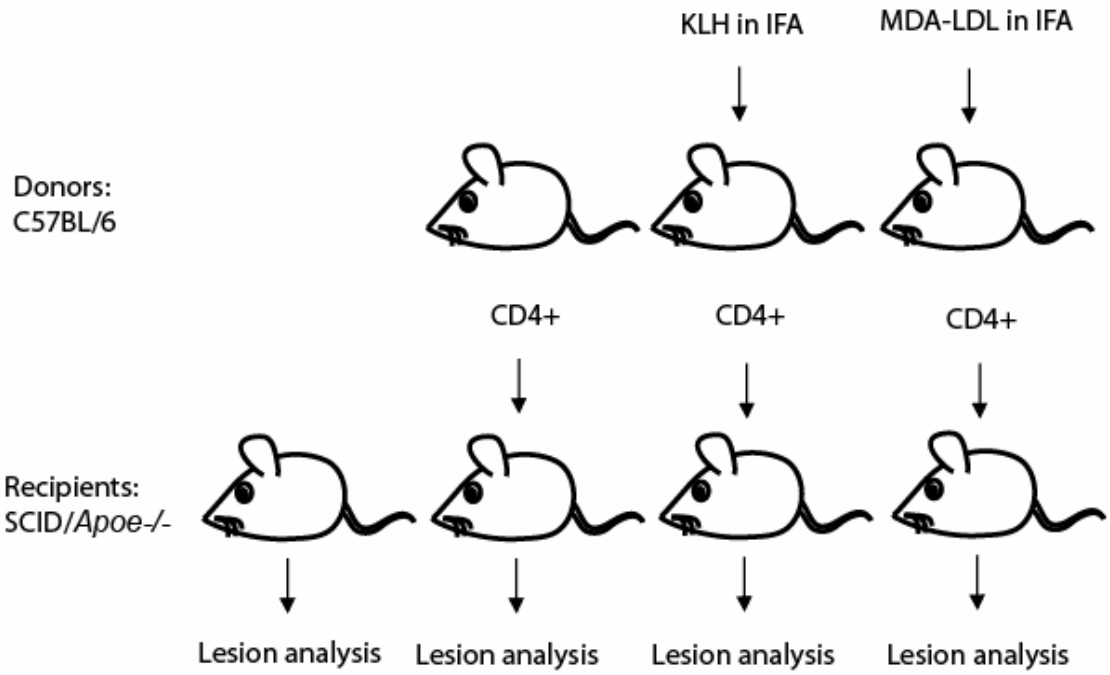

Figure 10. Experiment to investigate the role of $CD4^+$ T cells reactive to MDA-LDL.

It is known that Th1 responses dominate over Th2 responses in atherosclerosis. The Th1 cytokines IFNγ and TNFα are highly expressed in lesions whereas only low amounts of the Th2 cytokines IL-4, IL-5 and IL-10 can be found [204]. Inhibiting Th1 responses by administration of the drug pentoxifyllin to *Apoe*$^{-/-}$ mice or by genetic disruption of the Th1 driving transcription factor T-bet in *Ldlr*$^{-/-}$ mice reduces atherosclerosis [311, 312]. Furthermore, disruption of the Th2 signaling pathway in BALB/c mice, which are normally resistant to atherosclerosis, results in lesion development in these mice [146]. In *Apoe*$^{-/-}$ mice, circulating antibodies to oxLDL are mostly of the IgG2a isotype, typical of Th1 responses [203]. Interestingly, a switch from Th1 to Th2 antibodies against oxLDL occurs in *Apoe*$^{-/-}$ mice with severe hypercholesterolemia [203]. This phenomenon is possibly reflecting an increasing level of oxLDL in the lymphoid organs, since high concentrations of antigens usually induce Th2 responses while low antigen levels tend to elicit the Th1 pathway [313].

In the present study, the circulating level of IFNγ was increased in proportion to the acceleration of atherosclerosis, while IL-4 and IL-5 was undetectable, supporting the notion that IFNγ-producing Th1 cells play an important role in the disease process.

Another interesting finding in this study was that transfer of naïve $CD4^+$ T cells resulted in modestly increased lesions with signs of increased inflammation compared to untreated SCID/*Apoe*$^{-/-}$ mice. This may reflect the presence of a T cell population recognizing modified LDL or another pro-atherogenic antigen in the $CD4^+$ T cell preparation from non-immunized mice. Perhaps these clones are present in the donor, inhibited by peripheral tolerance, and are released from supression when transferred to an immunodeficient host. We found increased expression of VCAM-1 in the lesions after transfer of naïve $CD4^+$ T cells, suggesting that the transferred cells indeed enhanced local inflammation in the lesions. However, we did not detect any increase in systemic IFNγ in the mice receiving naïve T cells, suggesting that the inflammation was restricted to the lesions, or was of non-Th1 type. We did not measure any other cytokines than IFNγ in the sera, and it is possible that other cytokines might have been affected and the cytokine profile in the lesion might have been altered. Yet another alternative way for naïve T cells to contribute to atherogenesis is through lymphopenia induced homeostatic proliferation, generating cytokines and other immune interactions.

It has been shown in another study that immunization with MDA-LDL induces an antigen specific Th2 response characterized by IL-4, IL-5 and IL-13 [128]. In our study, T cells isolated from mice immunized with MDA-LDL responded to antigen re-challenge by producing IFNγ and TNFα, but no IL-5. The reason for this discrepancy is likely due to the differences in experimental design, for example in the number of injections, the dose of antigen injected, the route of injection, the culture medium and the concentration of antigen in the culture.

Since immunization with MDA-LDL decreases atherosclerotic lesion development in *Apoe*$^{-/-}$ mice [147, 171], it might appear puzzling that transfer of $CD4^+$ T cells from mice immunized with MDA-LDL aggravates disease as is shown in this study. However, the experimental setup used in this study excludes contribution from other cells such as B cells, $CD8^+$ T cells and APCs, which possibly could contribute to the positive effect of immunization. Especially B cells and antibodies are thought to contribute to the positive effect of immunization. This study evaluates only the role of antigen specific $CD4^+$ T cells in the absence of other

adaptive immune responses, and shows that adoptive transfer of purified $CD4^+$ T cells from MDA-LDL-immunized mice accelerates atherosclerosis. There is however still a possibility that subpopulations of $CD4^+$ cells, such as Treg, could be protective under certain circumstanses.

4.4 MDA-LDL PULSED DENDRITIC CELLS AGGRAVATE ATHEROSCLEROSIS

The accumulation of DC in atherosclerotic lesions suggests that DC might play a role in atherogenesis by initiating immune responses to plaque antigens in the local lymphoid tissues or by participating in antigen presentation in the lesions [194]. However, the role of DC for atherosclerosis-related autoimmunity has not yet been studied. To evaluate the role of DC in atherosclerosis, we pulsed bone marrow-derived DC with MDA-LDL and transferred these DC into *Apoe*$^{-/-}$ mice. The extent of disease was compared to that in animals treated with DC pulsed with KLH and to untreated animals (figure 11).

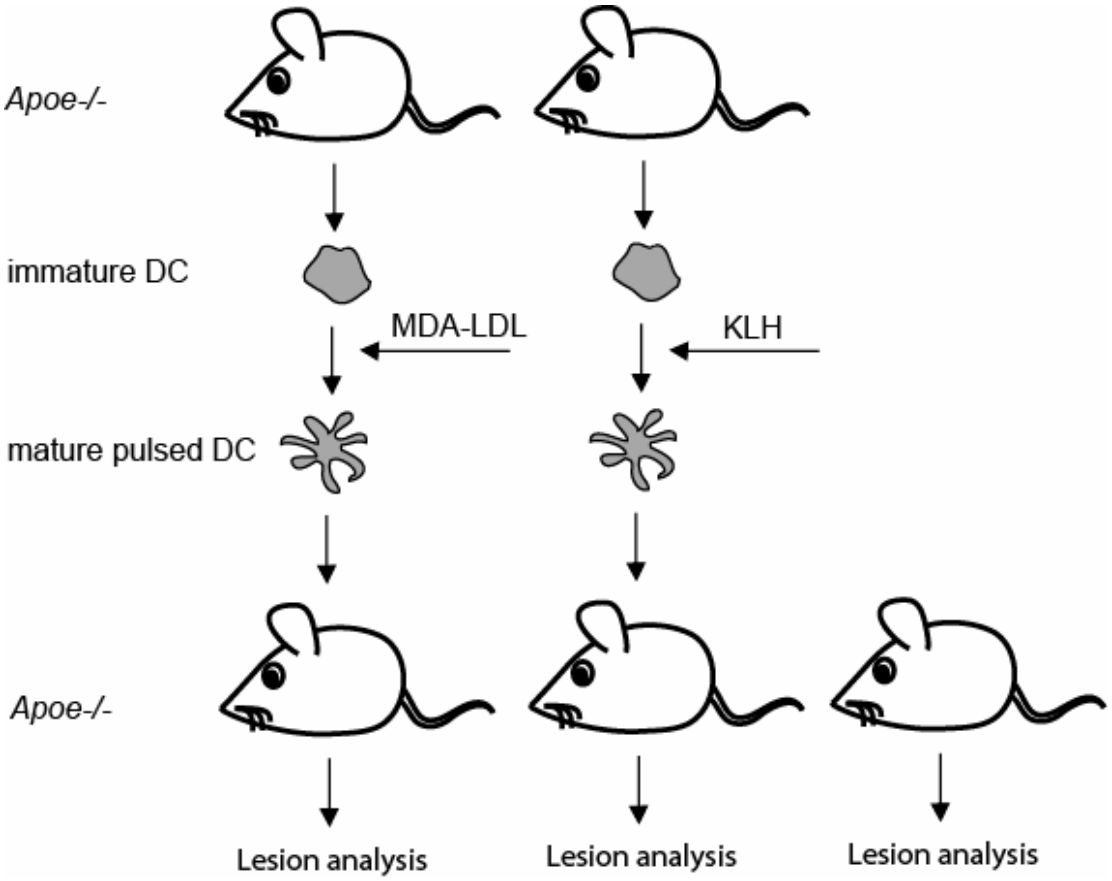

Figure 11. Outline of the experiment to study the role of DC in atherosclerosis.

Mice receiving MDA-LDL-pulsed DC showed significantly larger atherosclerotic lesions in the aortic root compared to untreated mice and to mice receiving KLH-pulsed DC. Our protocol including DC pulsed with antigen induced a strong immune response to the antigen, confirmed by circulating specific antibodies and a T cell proliferative response, but the immune response elicited by pulsed DC did not alter systemic inflammatory cytokines. Although administration of an irrelevant antigen, KLH, induced a strong systemic immune response to KLH, this immune reaction did not affect lesion development. Only transfer of MDA-LDL pulsed DC had effects on the local inflammation in the lesions, increasing expression of VCAM-1 and enhancing lesion progression. This finding illustrates that antigen specificity and the local inflammation are important in the development of atherosclerosis.

Several hypotheses about the life of a DC within the arterial wall have been proposed based on histological and experimental findings. It is reasonable to believe that VDC, as DC in other tissues, capture and process antigens, migrate to the regional lymph nodes and activate T cells. Analysis of lymph nodes draining the diseased aorta supports this idea, by showing that para-aortic lymph nodes attached to atherosclerotic vessels show higher numbers of mature DC than lymph nodes draining non-diseased vessels. It has been suggested that VDC screen the arterial wall for antigens and then migrate to the local lymphoid tissue where they maintain tolerance to self antigens and elicit immune reponses to harmful antigens. Our data show that DC indeed can take up and present MDA-LDL to the immune system, leading to pro-atherogenic immune reactions. This is the first experimental *in vivo* evidence for DC playing a role in atherosclerosis-related immunity.

In vitro studies have demonstrated that mildly oxidized LDL is able to induce maturation of DC from monocytes and from immature DC [314, 315]. To avoid the risk of having maturing effects of the antigen in the MDA-LDL group and not in the KLH group, we performed antigen pulsing in the presence of LPS. DC pulsed in the presence of LPS with MDA-LDL or KLH showed the same level of surface co-stimulatory molecules, indicating that they were in a similar stage of maturation. Our protocol thus involved maturation of DC by Toll-like receptor ligation, which is known to promote development of autoreactive T cells in the recipient [316, 317]. Our study suggests that activation of the immune system towards the auto-antigen MDA-LDL using LPS-activated pulsed DC aggravates atherosclerosis.

From immunization and cell transfer studies it is clear that the immune response to oxLDL has both protective and aggravating components and it remains to be investigated how to balance these atherogenic and atheroprotective responses. It is critical to explore how to use the inherent properties of the immune system to establish functional tolerance in a safe and efficacious manner. It is possible that treatment with immature DC may have a different effect on atherosclerosis than our protocol using mature DC. There are several strategies to generate immature tolerogenic DC, including culture in the presence of immuno-supressive cytokines and transfection with immuno-inhibitory genes [318-322]. Injection of immature DC may induce anergy or activate regulatory T cells, inducing antigen-specific tolerance *in vivo* [318-322]. Illustrating the importance of DC phenotype for the outcome of immunity to disease-related antigens, transfer of DC loaded with a myelin auto-antigen may either induce or protect from experimental autoimmune encephalomyelitis depending on the phenotype of the transferred DC [317]. Based on these findings, the possibility to induce immune tolerance to oxLDL through cultured DC should be explored in the future. The possibility to modulate the properties of an immune response by modifying DC phenotype may provide interesting opportunities for immunoprevention of atherosclerosis.

4.5 ATHEROPROTECTION BY IMMUNIZATION IN B CELL DEFICIENT MICE

Paradoxically, activation of the immune system towards modified LDL can be beneficial in atherosclerosis. Several studies have reported a 40-70% reduction in atherosclerosis after immunization with oxLDL, MDA-LDL or MDA-modified peptides from ApoB in hypercholesterolemic rabbits and mice [167-172]. Protective immunity seems to correlate with titers of anti-oxLDL IgG antibodies, suggesting that antibodies carry part of the protection achieved by immunization, and implying that antigen-specific $CD4^+$ T cells are activated and helping in the isotype-switch [121, 166, 171]. Interestingly, immunization is still protective in *Apoe*$^{-/-}$*/CD4*$^{-/-}$ mice, and still leads to increased levels of IgG antibodies specific for MDA-LDL, despite the absence of T cell-induced isotype-switch [147]. This suggests that production of some MDA-LDL specific IgG antibodies is independent of $CD4^+$ T cell help.

To study the role of B cells and antibodies in protective immunizations, we investigated lesion development in B cell-deficient μMT/*Apoe*$^{-/-}$ mice after immunization with MDA-LDL (figure 12). To be able to compare our results with

previous findings in *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*/CD4*$^{-/-}$ mice, we followed the same immunization protocol as in those studies. Our results show that immunization-induced atheroprotection remains also in the absence of B cells and antibodies, indicating that humoral immune responses are not obligatory for immunization induced atheroprotection and that other mechanisms than those mediated by antibodies are involved in the protection. We performed further experiments to investigate those B cell-independent mechanisms, and identified one possible explanation for atheroprotection by immunization, namely the induction of antigen specific regulatory T cells. When lymph node $CD4^+$ T cells from immunized *Apoe*$^{-/-}$ mice were re-challenged with antigen *in vitro*, we could detect an antigen specific expansion of $CD4^+/CD25^+/FoxP3^+$ T cells and elevated concentrations of IL-10 in the supernatant. We speculate that these regulatory T cells suppress oxLDL specific immune responses, thereby inhibiting inflammation and reducing atherosclerotic lesions in the immunized mice.

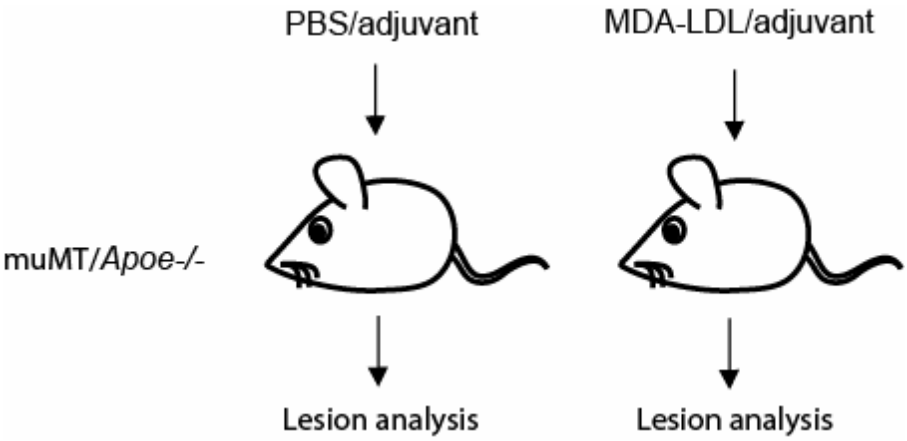

Figure 12. Experimental design to study the role of B cells and antibodies in protective immunization.

The outcome of immunization is, of course, determined by the antigen, but also by the adjuvant in which it is administered and the route, dose etc used for administration. Adjuvants are agents that enhance and prolong the immune response to the antigen by preventing catabolism of the immunogen and by activating immune cells. Alum is an adjuvant based on a gel containing aluminium salts, functioning simply by retaining the antigen at the injection site. Freund's complete adjuvant

(CFA) contains mineral oils and heat-killed mycobacteria promoting macrophage activation and inducing strong Th1 antibody responses. Incomplete Freund's adjuvant (IFA) lacks mycobacteria and induces antibodies without the strong T cell responses seen with CFA. Interestingly, several studies have incidentally discovered that injections of Freund's adjuvant or alum lead to atheroprotective effects despite the lack of any relevant antigen in the adjuvant mixture [127, 147, 292]. Attempts have been made to clarify the mechanism, but without conclusive results [323]. Interestingly, the protective effect of Freund's adjuvant is lost in $CD4^{-/-}$ mice, suggesting that $CD4^{+}$ T cells takes part in this protection. One can speculate that induction of immunosuppressing regulatory T cells could be one such mechanism, and our data support that hypothesis. Alternatively, the protection could be mediated by other mechanisms which could be impaired due to the lack of $CD4^{+}$ cells. Finding out the mechanism for the protective adjuvant effect is an important goal for future research since that knowledge could be useful when developing new therapies.

The results from the *$CD4^{-/-}$*mice and the μMT mice imply a complexity in the protective mechanisms induced by immunization, suggesting that several mechanisms and cell types are involved, perhaps both from innate and adaptive immunity. Absence of $CD4^{+}$ T cells or B cells is clearly not enough to ameliorate the protective effect.

5 CONCLUDING REMARKS

We have utilized mouse models of atherosclerosis to unravel immune mechanisms involved in the progression of and protection against atherosclerosis. The immunomodulatory studies included in this thesis have contributed to the knowledge about the role of immune reactions in atherosclerosis by filling some gaps, extending some concepts and introducing a few new principles. This field of research is constantly growing, and it is clear that the immune response in atherosclerosis is complex and can both enhance and inhibit the disease. Thus follows that the interpretation of immunomodulatory experiments in atherosclerotic models is complicated, and the expected results can easily turn out to be unexpected.

Our comparison of immune responses in *Apoe*$^{-/-}$mice and wild-type mice in **paper I** show that apoE controls T cell activation by down-regulating the expression of MHC class II and costimulatory molecules on the antigen-presenting cell. We thereby give an explanation to why apoE can inhibit T cell proliferation and we also provide new insights into the phenotype of *Apoe*$^{-/-}$mice.

By utilizing immunodeficent SCID/*Apoe*$^{-/-}$ mice, we in **paper II** extend the principle of IL-18 as a pro-atherogenic cytokine and demonstrate that the disease-aggravating effect of IL-18 occurs also in the absence of T cells. We thereby contribute to the knowledge about the role of pro-inflammatory cytokines in atherosclerosis and illustrate that NK cells, macrophages and vascular cells produce IFNγ in response to IL-18 in amounts that are sufficient for disease progression.

We show in **paper III** that transfer of MDA-LDL specific $CD4^+$ T cells to SCID/*Apoe*$^{-/-}$ mice accelerates atherosclerosis. This supports the notion that Th1 cellular immunity is pro-atherogenic and identifies MDA-LDL as an autoantigen that promotes atherosclerosis in hypercholesterolemic mice.

In paper IV we demonstrate that DC presenting MDA-LDL induce antigen-specific pro-atherogenic immunity that augments local inflammation and growth of atherosclerotic lesions in *Apoe*$^{-/-}$ mice. We conclude that DC capture and presentation of oxLDL might take part in the initiation of atherosclerosis-related immunity.

We have recently shown that *Apoe*$^{-/-}$*CD4*$^{-/-}$ mice are protected from atherosclerosis by immunization with MDA-LDL [147], confirming our previous finding that immunization with MDA-LDL inhibits disease development [171]. In

paper V we demonstrate B cell-independent protection by MDA-LDL immunization in μMT/*Apoe*$^{-/-}$ mice, and identify a possible protective mechanism involving induction of regulatory T cells.

The effects of immune responses to modified LDL are multiple and complex. Since immunization with modified LDL enhances several mechanisms within both cellular and humoral immune responses, and perhaps even innate immune responses, it is possible that some of the mechanisms activated by immunization are deleterious, whereas others are protective. The timing for the induction of such reactions may also be relevant, as well as the form in which the antigen is presented. The contribution of this thesis to the knowledge about the balance between protective and deleterious immune mechanisms in atherosclerosis is summarized in figure 13. Still we do not fully understand the factors leading to these qualitatively different immune responses. Further studies are necessary to explore how the balance between protective and aggravating immune responses can be controlled in atherosclerosis. Until then, safe and effective immunomodulation in patients with atherosclerosis remains a hope for the future. Antibodies to oxLDL is one promising strategy for therapeutic immunomodulation, and regulatory T cells are emerging as another. However, one must keep in mind that most successful immunomodulatory studies have been performed in young mice, without established lesions when the treatment started. Even if prevention of atherosclerosis is interesting in the longer perspective, the most urgent task is to find treatments that can induce regression of established lesions. Further studies are needed to address the question if immunomodulation can work also on already established atherosclerosis and promote regression of the lesions. And due to the complex etiology of the disease, immunomodulation will probably never completely prevent development of atherosclerosis, but could be a complement to other treatments.

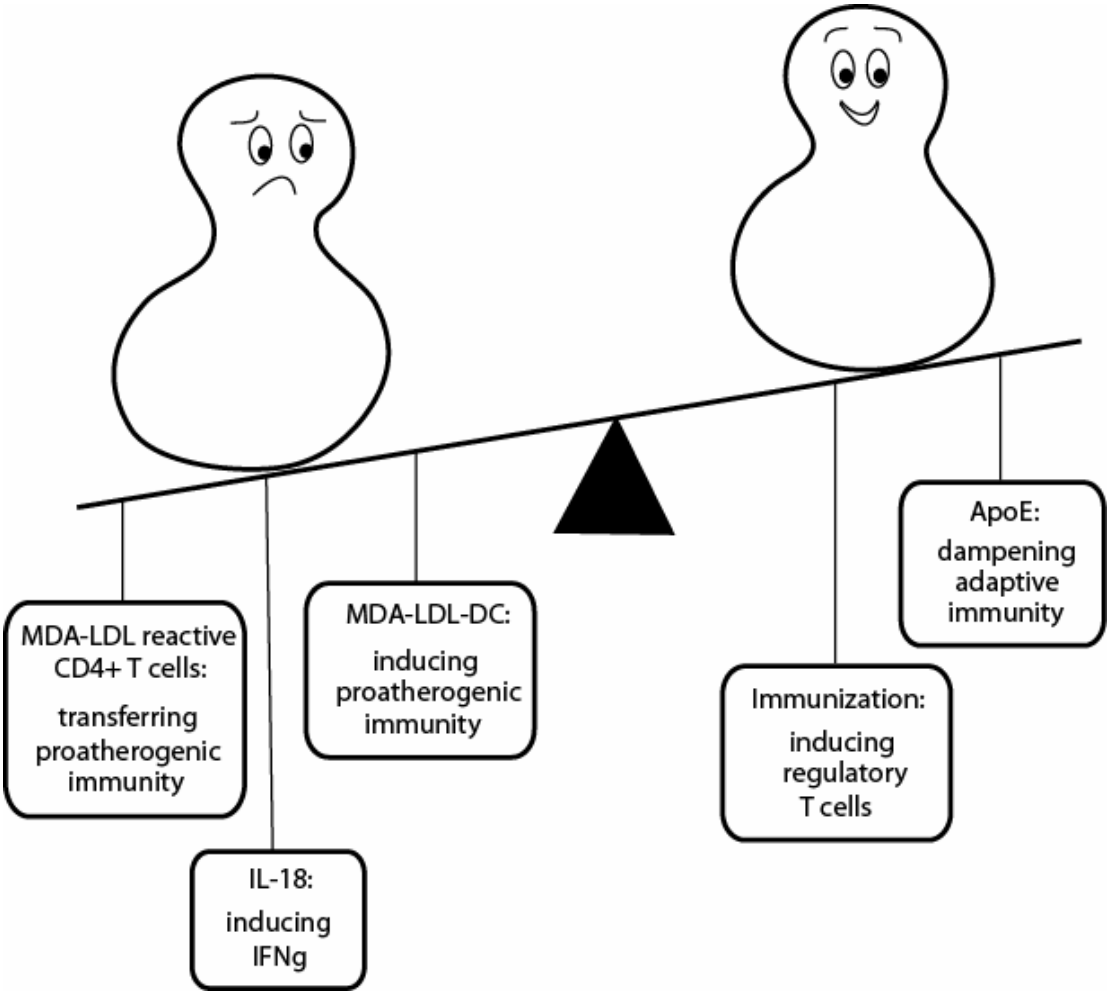

Figure 13. Protective and aggravating immunomodulation described by the studies in this thesis.

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