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# **EFFECTOR MECHANISMS OF IMMUNITY IN ATHEROSCLEROSIS**

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Till Mamma och Pappa



## ABSTRACT

Atherosclerosis is a disease of the medium and large sized arterial vessels, characterized by cholesterol deposition, inflammation and fibrosis. Low-density lipoprotein (LDL) particles carrying cholesterol are trapped within the arterial wall. LDL has been shown to be a candidate antigen for immune responses associated with atherosclerosis and elicits both innate as well as adaptive immune responses. These studies have used different mouse models to unravel immune mechanisms involved in the progression and protection of disease, and can lead to new therapeutic opportunities.

To explore the role of dendritic cells (DCs) in atherogenesis, in the context of an atherosclerosis-related antigen, we injected hypercholesterolemic *ApoE*<sup>-/-</sup> mice with malondialdehyde (MDA)-LDL pulsed DCs. This cell transfer induced immune responses specific for components of LDL that augmented local inflammation in the vessel wall and accelerated growth of atherosclerotic lesions. This study shows that DCs presenting the autoantigen LDL can augment atherogenesis.

DCs can be conditioned into a tolerogenic state by immunomodulatory mediators. HuB100<sup>tg</sup>*Ldlr*<sup>-/-</sup> mice were injected with DCs that had been made tolerogenic by treatment with IL-10 and loaded with the protein moiety of LDL, apolipoprotein B100 (ApoB100). One single injection led to a significant reduction of atherosclerotic plaque burden in the aorta with decreased lesional as well as systemic inflammation. This DC therapy diminished the autoreactive T cell response to ApoB100, showing that tolerogenic DC presenting the protein part of LDL can attenuate atherosclerosis.

Animals that are immunized with LDL together with adjuvant are protected against atherosclerosis, but the underlying mechanisms remain unknown. *Rag2*<sup>-/-</sup>/*ApoE*<sup>-/-</sup> mice, lacking functional T as well as B cells, and B cell-deficient *μMT*/*ApoE*<sup>-/-</sup> mice were immunized with homologous oxLDL. Adaptive immunity was shown to be obligatory for immunization-induced atheroprotection while humoral immunity was dispensable.

Degradation of extracellular matrix (ECM) in the vessel wall is central to inflammatory vascular diseases. SerpinA3 is an inhibitor of several immune cell-derived proteases involved in this process. SerpinA3 expression was detected in human atherosclerotic lesion, while its expression was decreased in human aortic abdominal aneurysm (AAA). Overexpression of the murine orthologue serpinA3n had no effect on atherosclerotic lesion size in *ApoE*<sup>-/-</sup> mice, but inhibited CaCl<sub>2</sub>-induced aneurysm formation.

In conclusion, we have studied the role of effector mechanisms of different immune cells in the pathological process of atherosclerosis. Some of the findings may have applications in the clinic, as novel therapeutic targets for cardiovascular disease. In brief, we found that LDL or components thereof displayed by immunogenic DCs aggravate atherosclerosis, while their presentation by tolerogenic DCs ameliorates disease. Protective immunization is dependent on adaptive, but not humoral immunity and serpinA3 plays a role in inflammatory vascular disease.

## LIST OF PUBLICATIONS

This thesis is based on the following papers, referred to in the text by their corresponding roman numerals (I-IV).

- I. Hjerpe C, **Johansson D**, Hermansson A, Hansson GK, Zhou X. Dendritic cells pulsed with malondialdehyde modified low density lipoprotein aggravate atherosclerosis in *Apoe*<sup>-/-</sup> mice. *Atherosclerosis*. 2010 Apr;209(2):436-41
- II. Hermansson A, **Johansson D**, Ketelhuth DFJ, Andersson J, Zhou X, Hansson GK. Immunotherapy with tolerogenic apolipoprotein B-100-loaded dendritic cells attenuates atherosclerosis in hypercholesterolemic mice. *Circulation*. 2011 Mar 15;123(10):1083-91.
- III. **Johansson D**, Hjerpe C, Robertson AK, Arnal JF, Hansson GK, Zhou X. Adaptive immunity is obligatory but humoral immunity is dispensable for immunization-induced atheroprotection. *Submitted for publication*.
- IV. Wågsäter D, **Johansson D**, Fontaine V, Vorkapic E, Bäcklund A, Razuvaev A, Mäyränpää MI, Hjerpe C, Caidahl K, Hamsten A, Franco-Cereceda A, Wilbertz J, Swedenborg J, Zhou X, Eriksson P. Serine protease inhibitor A3 in atherosclerosis and aneurysm disease. *Submitted for publication*.

Note: In paper I, III, and IV the two first authors contributed equally. In paper IV, the two last authors share senior authorship.

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## LIST OF ABBREVIATIONS

|              |  |
|--------------|--|
| APC          | Antigen presenting cell                        |
| ApoB         | Apolipoprotein B                               |
| ApoE         | Apolipoprotein E                               |
| BCR          | B cell receptor                                |
| cAMP         | Cyclic adenosine monophosphate                 |
| CAD          | Coronary artery disease                        |
| CCL          | Chemokine (C-C motif) ligand                   |
| CD           | Cluster of differentiation                     |
| CDP          | Common dendritic cell precursor                |
| CFA          | Complete Freund's adjuvant                     |
| CPM          | Counts per minute                              |
| CRP          | C-reactive protein                             |
| CTL          | Cytotoxic T lymphocyte                         |
| CTLA         | Cytotoxic T-lymphocyte antigen                 |
| CXCL         | C-X-C motif chemokine ligand                   |
| CVD          | Cardiovascular disease                         |
| DC           | Dendritic cell                                 |
| DAMPs        | Damage-associated molecular patterns           |
| DNA          | Deoxyribonucleic acid                          |
| EC           | Endothelial cell                               |
| ECM          | Extracellular matrix                           |
| Fab          | Fragment antigen-binding                       |
| Fc           | Fragment crystallizable                        |
| FoxP         | Forkhead box P                                 |
| GC           | Germinal center                                |
| GM-CSF       | Granulocyte-monocyte colony-stimulating factor |
| HMG-CoA      | 3-hydroxy-3-methylglutaryl coenzyme A          |
| HSP          | Heat-shock protein                             |
| ICAM         | Intracellular adhesion molecule                |
| IFA          | Incomplete Freund's adjuvant                   |
| IFN $\gamma$ | Interferon gamma                               |
| Ig           | Immunoglobulin                                 |
| IL           | Interleukin                                    |
| iTreg        | Inducible T regulatory cell                    |
| KLH          | Keyhole-limpet hemocyanin                      |
| LDL          | Low-density lipoprotein                        |
| LDLR         | Low density lipoprotein receptor               |
| LPS          | Lipopolysaccharide                             |
| M-CSF        | Macrophage colony-stimulating factor           |
| MCP          | Monocyte chemotactic protein                   |
| MDP          | macrophage and dendritic cell progenitor       |
| M-CSF        | Macrophage colony-stimulating factor           |
| MDA          | Malondialdehyde                                |
| MHC          | Major histocompatibility complex               |



|          |  |
|----------|--|
| MI       | Myocardial infarction                  |
| MMP      | Matrix metalloproteinase               |
| mRNA     | messenger ribonucleic factor           |
| NK cell  | Natural Killer cell                    |
| NKT cell | Natural Killer T cell                  |
| nTreg    | Natural T regulatory cell              |
| OD       | Optical density                        |
| oxLDL    | Oxidized low density lipoprotein       |
| PAMPs    | Pathogen-associated molecular patterns |
| PCR      | Polymerase chain reaction              |
| PRR      | Pattern recognition factor             |
| RA       | Rheumatoid arthritis                   |
| Rag      | Recombination-activating gene          |
| SCID     | Severe combined immunodeficiency       |
| ScR      | Scavenger receptor                     |
| SHM      | Somatic hypermutation                  |
| SLE      | Systemic lupus erythematosus           |
| SMC      | Smooth muscle cell                     |
| SR-A     | Scavenger receptor A                   |
| T-bet    | T-box expressed in T cells             |
| 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                      |
| TNF      | Tumor necrosis factor                  |
| TNFSF    | Tumor necrosis factor superfamily      |
| VCAM     | Vascular cell adhesion molecule        |
| VLDL     | Very low-density lipoprotein           |



# 1 INTRODUCTION

Atherosclerosis is a slowly progressive chronic disorder of large and medium-sized arteries, and the main underlying pathological process of cardiovascular diseases, such as coronary artery disease and cerebrovascular disease. Cardiovascular disease is today one of the leading causes of death and loss of productive life years globally.

It was for many years believed that atherosclerosis was merely passive accumulation of cholesterol in the vessel wall. Today, the picture is much more complex, with atherosclerosis being thought of as a chronic inflammatory disease. The understanding that immune mechanisms play a decisive role in atherosclerosis has focused our attention on the immune system as a possible novel target in prevention and treatment of cardiovascular disease.

This thesis is focused on the role of different immune cells, as well as proteases that they secrete, in the pathological process of atherosclerosis. Some of the findings uncover mechanisms involved in the disease, while others illustrate potential immunomodulatory strategies for atherosclerosis treatment.

An overview of the immune system is provided, followed by current knowledge of its role in atherosclerosis. An emphasis is put on cells and molecules important for the studies. In the end the importance of my own projects is discussed in the light of that knowledge.

## 1.1 OVERVIEW OF THE IMMUNE SYSTEM [1]

The main function of the immune system is defense against harmful material and infectious microbes, such as bacteria, viruses, fungi and parasites. The response of the immune system to microbes or other substances is referred to as *immune responses* and the protection achieved as *immunity*. An *antigen* is a substance that induces an immune response. Defense against microbes is mediated by the early reactions of innate immunity and the later responses of adaptive immunity. The cells and molecules of innate and adaptive immunity cooperate efficiently and communicate by cell-to-cell contact using surface molecules and by secreting soluble messengers called cytokines. Inflammation consists of recruitment of leukocytes (immune cells) and plasma proteins into a site of infection, and the activation of the leukocytes and proteins to eliminate the infectious agent.

### 1.1.1 Innate immunity

Innate immunity is the initial responses to microbes. It consists of epithelial barriers, circulating and tissue cells, as well as plasma proteins. These defense mechanisms exist before an encounter with microbes, thereby allowing rapid activation.

Some components of innate immunity, such as epithelial barriers, are functioning at all times, whereas other components of innate immunity require activation upon microbe recognition. This is mediated through recognition of structures that are characteristic of microbial pathogens (nonself), but not by structures present on mammalian cells (self). The microbial substances that stimulate innate immunity are called pathogen-associated molecular patterns (PAMPs), and the receptors that bind these conserved structures are called pattern recognition receptors (PRRs). In addition, PRRs can recognize endogenous ligands from host cellular debris from injured or dying tissue, termed damage-associated molecular patterns (DAMPs) [2, 3].

The PRRs are encoded in germline DNA and recognize only a limited number (about  $10^3$ ) of patterns. Some PRRs, such as the toll like receptors (TLRs), transmit activating signals that promote inflammatory signals. Other PRRs, such as the scavenger receptors (ScRs), mainly participate in the uptake of microbes into phagocytes.

#### 1.1.1.1 Circulating proteins of innate immunity

Several different soluble proteins found in plasma and extracellular fluids bind PAMPs and DAMPs and serve as effector molecules of the innate immune system. The major components are the complement system, the collectins, the pentraxins, and the ficolins.

The complement system identifies microbes either by direct recognition of certain microbial surface structures, or by detection of antibodies bound to the microbe. This initiates an inflammatory process and promotes ingestion (phagocytosis) and lysis of microbes.

The most common biomarker of inflammation measured in the clinic is C-reactive protein (CRP). CRP belongs to the pentraxin family and its expression in the liver is induced by leukocyte-derived cytokines such as interleukin-1 (IL-1) and IL-6. CRP levels are very low in healthy individuals, but can increase up to 1000-fold in response to inflammatory stimuli.

#### 1.1.1.2 *Cells of innate immunity*

The most numerous effector cells of the innate immune system are bone marrow-derived cells that circulate in the blood and migrate into tissues, by a process called extravasation. Extravasation is induced by in tissues activated macrophages, endothelial cells (ECs) and smooth muscle cells (SMCs) producing chemoattractants and cytokines. Cytokines, such as tumor necrosis factor (TNF) and IL-1, induce endothelial expression of adhesion molecules, e.g. E- and P-selection, and integrins, such as vascular cell adhesion molecule 1 (VCAM-1) and intracellular adhesion molecule 1 (ICAM-1). This results in binding of leukocytes to endothelium, and their migration into the tissue is subsequently activated by chemoattractants.

##### 1.1.1.2.1 Innate like lymphocytes

T and B lymphocytes are the cells of adaptive immunity, but certain subsets of lymphocytes have very little diversity in their antigen receptors and are more related to the effector cells of innate immunity. They recognize PAMPs and are mostly located in barrier epithelia and serosal cavities. Intraepithelial T cells are present in the epidermis of the skin and in mucosal epithelia. The peritoneal cavity contains a population of innate B lymphocytes, called B-1 cells. B-1 cells produce molecules, called natural antibodies, which often bind structures shared by many types of bacteria, such as lipopolysaccharide (LPS).

##### 1.1.1.2.2 Natural Killer cells

Natural killer cells (NK cells) are a lineage of cells related to lymphocytes that recognize cells with reduced expression of MHC class I, and respond by directly killing these cells and by secreting inflammatory cytokines. They are a major source of the cytokine interferon gamma (IFN $\gamma$ ), which activates macrophages to kill ingested microbes.

##### 1.1.1.2.3 Mast cells

Mature mast cells are found throughout the body and are involved in immune responses to parasites and are mediating allergic reactions. Bone marrow progenitors migrate to the peripheral tissues as immature cells and undergo differentiation *in situ*. There are two major subsets of mast cells; the mucosal mast cells and the connective tissue mast cells. Activation of mast cells results in secretion of their granula content, synthesis and secretion of lipid mediators, and synthesis and secretion of cytokines

##### 1.1.1.2.4 Phagocytes

Phagocytes, including neutrophils and macrophages, are cells whose primary function is to identify, ingest (phagocyte), and destroy harmful material such as microbes, dead cells and debris. In addition, phagocytes produce cytokines important both for innate and adaptive immune responses.

Polymorphonuclear neutrophils are the most abundant population of circulating white blood cells and mediate the earliest phases of inflammatory responses. Their production is stimulated by granulocyte colony-stimulating factor (GM-CSF), and bone marrow-

derived neutrophils may migrate from bone marrow to sites of infection within a few hours after the entry of microbes, where they function for a few hours and then die. Neutrophils have granules, which contain presynthesized enzymes, such as elastase, and other microbicidal substances. These will come in contact with the microbes upon the formation of the phagosome or when the activated neutrophils secrete their granula content.

The cells of the mononuclear phagocyte system originate in the bone marrow, circulate in the blood, and mature and become activated in various tissues. Once monocytes enter tissues, they mature and differentiate into macrophages. Macrophages typically respond to microbes nearly as fast as neutrophils do, but macrophages survive much longer at sites of inflammation. Unlike neutrophils, macrophages are not terminally differentiated and can undergo cell division at an inflammatory site.

#### 1.1.1.2.5 Dendritic cells

Dendritic cells (DCs), first described by Ralph Steinman in the 1970s, play important roles in innate responses to infections and other harmful material [4]. They are specialized in antigen capture as immature cells by processes such as uptake by ScRs and macropinocytosis [5]. DCs also express PRRs, and respond to microbes by secreting cytokines. DCs are widely distributed in lymphoid tissues, mucosal epithelium, and organ parenchyma. They are derived from the macrophage and DC progenitor (MDP) in bone marrow. The MDPs give rise to both monocytes and the common DC precursor (CDP). CDPs give rise to DCs, which migrate to the periphery where they populate almost all organs and differentiate into several different subsets of DCs. Some of the monocytes that enter tissues can differentiate into so called monocyte-derived DCs [6].

The literature suggests the existence of a number of unique DC subsets, which differ in cell-surface markers, anatomic location, and function. Heath et al argues for a more generalized model where DCs can be divided into four subgroups, e.g. CD11b<sup>+</sup> DCs, CD11b<sup>-</sup> DCs, and monocyte-derived inflammatory DCs [7]. All myeloid DC express the integrin CD11c, however CD11c can be expressed by other cell types, and there is no single cell-surface antigen that identifies all DCs [7]. The fourth subgroup consists of cells with plasma cell-like morphology called plasmacytoid DCs (pDCs) that produce large amount of IFN $\alpha$  and  $\beta$  in response to viral and bacterial stimuli [8-10].

Activated DCs undergo both morphological and phenotypical changes leading to functional maturation. Changes in expression of chemokine receptors and adhesion molecules results in migration of the DCs to peripheral lymphoid organs [11]. DC maturation also leads to decreased uptake function, while the ability for antigen presentation is increased. The activated DCs enter the T cell areas of lymphoid organs where they play an important role in initiating T immunity and serve a critical function in linking innate and adaptive immune responses [12].

### 1.1.2 Adaptive immunity

Adaptive immune responses are more potent and specialized, and are able to eliminate microbes that resist the defense mechanisms of innate immunity. The adaptive immune system is able to recognize and react to a larger number of microbial and nonmicrobial substances. In addition, its antigen receptors have an extraordinary capacity to distinguish between different, even closely related, molecules, and for this reason it is also called specific immunity. It also has an ability to “remember” and respond more vigorously to repeated exposures to the same microbe.

The main components of adaptive immunity are cells called lymphocytes and their secreted products. Lymphocytes are developed in the primary lymphoid organs; the bone marrow and the thymus. Naïve lymphocytes then circulate the periphery and are activated upon antigen encounter in the secondary / peripheral lymphoid organs; lymph nodes, spleen, mucosal and cutaneous immune systems.

The genes encoding the antigen receptors in lymphocytes are formed by recombination of DNA segments during the maturation of these cells, resulting in the generation of millions of different receptors and a highly diverse repertoire of antigen specificities among different clones of lymphocytes. Each clone expresses antigen receptors of the same specificity that are different from the receptors of other clones. The antigen receptors can recognize different parts of a single antigen, called epitopes and the lymphocyte clones can discriminate between  $10^7$ - $10^9$  different epitopes within one individual.

There are two types of adaptive immune responses, called humoral immunity and cellular immunity, which are mediated by different components of the immune system and function to eliminate different types of microbes.

#### 1.1.2.1 Humoral Immunity

Humoral immunity is mediated by molecules in the blood and mucosal secretions, called antibodies or immunoglobulins (Ig), which are produced by lymphocytes called B cells. It is the principal defense mechanism against extracellular microbes and their toxins because secreted antibodies can bind and assist in their elimination.

The major subsets of B cells are follicular B cells, marginal zone B cells, and B-1 B cells, each of which is found in distinct anatomical locations within lymphoid tissues. They partially mature in the bone marrow, enter the circulation, and populate the peripheral lymphoid organs where they complete their maturation. To avoid formation of potentially dangerous autoantibodies, B cells that bind with high affinity to self-antigens go into apoptosis, a process called negative selection.

Naïve B cells require two signals for activation. Antigen binding by the B cell receptor (BCR), which is a membrane bound antibody, delivers signal 1. Signal 2 is co-stimulation (the most important being CD40 – CD40 ligand) from a T cell that has recognized the same antigen. Certain immunogenic antigens can activate a B cell without T cell help (T-independent antigens) [13].

Upon activation, B cells proliferate and differentiate. Some of the progeny of the expanded B cell clones differentiate into antibody-secreting plasma cells. Each plasma cell secretes antibodies that have the same antigen binding site as the BCR that initially recognized the antigen. The antigen binding site of the BCR is in the variable region of the N-terminal Fab domain of the antibody. The C-terminal Fc chain determines the effector function, and can be exchanged in an activated B cell in a cytokine dependent manner (isotype switch).

Mammalian B cells are able to produce several isotypes (or classes) of antibodies. IgM, and IgD are the first isotypes synthesized upon B cell activation, but a subsequent stimulation can induce isotype switch resulting in expression of IgG, IgE, or IgA. Each Ig isotype is specialized for particular modes of antigen removal. IgM activates complement whereas IgG, the most abundant isotype in serum, binds receptors on phagocytic cells. Importantly IgG antibodies can also cross the placenta to provide maternal protection to the fetus. IgA antibodies are instead more abundant in secretions, such as tears and saliva where they coat invading pathogens to prevent proliferation. IgE antibodies bind basophils and mast cells to activate histamine release and are involved in allergy and protection against parasites [14].

Some of the activated B cells undergo somatic hypermutation (SHM), a process in which point mutations are introduced in the variable region of the BCR. This takes place in compartments of lymphoid organs called germinal centers (GC). After SHM, B cell clones undergo clonal selection, by which clones expressing high-affinity antibodies are selected in a process termed affinity maturation. Thereafter, B cells emigrate from the follicle and differentiate into long-lived plasma cells and memory B cells. Memory B cells have extremely long lives (years) and maintain the ability to respond rapidly to antigen re-exposure by differentiating into plasma cells [14, 15].

#### *1.1.2.2 Cellular Immunity*

Intracellular microbes, such as viruses and certain bacteria, can sometimes survive and proliferate inside phagocytes and other host cells, where they are inaccessible to circulating antibodies. Defense against such infections is a function of cellular immunity, which promotes the destruction of intracellular microbes through killing the infected cells and thereby eliminating reservoirs of infection and pathogen spread.

Cellular immunity is mediated by T lymphocytes. After leaving the bone marrow, T cells first mature completely in the thymus and then enter the circulation to populate peripheral lymphoid tissues. The two major T cell subsets are CD4<sup>+</sup> helper T lymphocytes (Th cells) and CD8<sup>+</sup> cytotoxic T lymphocytes (CTLs). Both cell types express an antigen receptor called the T cell receptor (TCR) that is a heterodimer composed of either  $\alpha\beta$  or  $\gamma\delta$  chains. T cells also express the pan-T cell marker CD3 that together with the TCR dimer and the co-receptors (CD4 or CD8) forms the TCR complex.

In response to antigenic stimulation, CTLs kill cells displaying particles recognized as non-self, such as virus infected cells and tumor cells. Activated Th cells secrete cytokines that stimulate the proliferation and differentiation of themselves. The Th cell-derived cytokines also activate other cells, including B cells, macrophages, and other



leukocytes. Activated Th cells differentiate into several different subpopulations, depending on the local environment. The first and best characterized subsets are the Th1 and Th2 cells [16]. Several other subsets with different functions and cytokine patterns (regulatory T cells, Th17, Th9, T follicular helper cells) have since then been described [17] [18-20].

#### 1.1.2.2.1 Antigen presentation

T cells have a restricted specificity for antigens; they recognize only denatured, unfolded peptides presented by specific surface proteins of other cells, called major histocompatibility (MHC) molecules. As a result, T cells recognize and respond to cell surface-associated but not soluble antigens. A structure, called the immunological synapse, forms between the T cell and the antigen presenting cell (APC) during the recognition of the MHC:peptide complex by the TCR [21]. To fulfill their physiological function, MHC proteins must first acquire their peptide antigens, a function that is executed differently by two structurally distinct types of MHC molecules; class I and class II [22].

MHC class I molecules, present on most nucleated cells, report intracellular events (such as viral infection, the presence of intracellular bacteria or cellular transformation) to CD8<sup>+</sup> T cells. The peptides in the peptide-binding groove of MHC class I molecules are derived from cytosolic proteins, degraded by the proteasome complex, and are 8-11 amino acids long. For MHC class II molecules, the goal is to sample the extracellular milieu and present antigens to CD4<sup>+</sup> T cells. The molecular expression of MHC class II molecules is mostly restricted to professional APCs, including macrophages, B cells, DCs and thymic epithelial cells. After internalization by APCs, the exogenous antigens are degraded in endocytic vesicles generating a large pool of peptide antigens. In contrast to MHC class I, the peptide-groove of the MHC class II molecule is open, resulting in display of larger peptides, usually 13-17 amino acids long [23].

Certain T cell subtypes also recognize antigens presented on the molecule CD1, which is a MHC-like molecule mostly restricted to APCs. In contrast to MHC molecules that present peptides, it can instead display a broad range of lipid antigens to specific CD1-restricted T cells. Invariant NKT (iNKT) cells, expressing surface molecules characteristic of both NK cells and T cells, are the most extensively CD1-restricted T cells studied [24].

Among the APCs, DCs are the best activators of naïve T cells. They are two orders of magnitude more potent than other cells in eliciting specific T-cell effector responses [25]. Immature DCs are found in most tissues where they engulf antigens and migrate to draining lymph nodes. PRR ligation induces maturation of a DC leading to a cell with less ability of uptake and increased capacity to stimulate and present antigens to T cells. This is achieved by higher expression of MHC molecules, combined with upregulation of co-stimulatory molecules and secretion of inflammatory cytokines.

#### 1.1.2.2.2 Co-stimulation

When the MHC:peptide complex is transported to the APC surface it can interact with T cells via the TCR and become activated, thereby receiving signal 1. However,

specific antigen recognition by the TCR is not enough for initiation of T cell responses. Accessory molecules, that facilitate signaling by the TCR complex, provide “second signals” to fully activate T cells. These are called co-stimulatory molecules and the most studied are the B7.1 (CD80) and B7.2 (CD86) molecules, expressed by APCs, that bind to the CD28 molecule on T cells [26]. The interaction delivers signals to the T cells that induce expression of anti-apoptotic proteins, production of growth factor and cytokines, and promote T cell proliferation and differentiation. The most important growth factor released by T cells is IL-2 that acts in an autocrine fashion, by binding to the IL-2R, to further clonally expand the T cells [27].

Another important family of co-stimulatory molecules, upregulated after T cell activation, is the tumor necrosis factor superfamily (TNFSF), including CD137/CD137L, OX40/OX40L, CD30/CD30L, CD27/CD70, and CD40/CD40L. CD40L binding to CD40 leads to activation of the responding cell including antibody production of B cells and increased destruction of phagocytosed microbes in macrophages [28].

#### 1.1.2.3 Immune tolerance

Reactions that can cause damage to the host, such as exaggerated immune responses to microbes or destruction by self-reactive lymphocytes, have to be regulated. Tolerance to self-antigens is a fundamental property of the normal immune system, and failures thereof cause *autoimmunity*.

One mechanism of self-tolerance or excessive immune responses to foreign is determined in a cell-intrinsic manner. For example, some lymphocytes are programmed to die by apoptosis when exposed to self-antigens during their development in the primary lymphoid organs (*central tolerance*) [29]. Those that have escaped clonal deletion in the bone marrow and thymus can be rendered anergic (functionally inactivated) upon exposure to self-antigen in the periphery (*peripheral tolerance*). Activation-induced cell death, inhibitory receptors and negative signaling molecules may also contribute to increased activation thresholds or decreased survival of lymphocytes.

Another mechanism is cell-extrinsic; the normal immune system produces a population of T cells, called regulatory T cells (Tregs), that actively keep in check the activation and expansion of aberrant or overreactive lymphocytes [17]. Tregs produced in the thymus, CD4<sup>+</sup> natural Tregs (nTregs), are characterized by their expression of the transforming growth factor- $\beta$  (TGF $\beta$ )-regulated transcription factor forkhead box P3 (FoxP3), and many of them also express the IL-2 receptor  $\alpha$ -chain (CD25) [30]. Tregs induced from naïve FoxP3<sup>+</sup>CD25<sup>+</sup>CD4<sup>+</sup> T cells in the periphery (iTregs) can also acquire FoxP3 expression and consequently regulatory function. In addition to nTregs and iTregs, other types of regulatory T cells have been described that do not require FoxP3 expression; Th3 cells, CD8<sup>+</sup>CD103<sup>+</sup> suppressor T cells, and IL-10 producing Tr1 cells [31-37].

Treg-mediated suppression can be either antigen-specific or non-specific, and the target cell is usually other T cells. Tregs regulate immune responses by secreting inhibitory cytokines and molecules, such as TGF $\beta$ , IL-10, galectin and cAMP [38-41]. Through

their high expression of CD25 they can starve T effector cells by depriving them of IL-2 that is needed for survival [42]. Tregs can also suppress APC function via downregulation of co-stimulatory molecules or by inducing immunoinhibitory molecules, for example through binding to CD80/86 with the inhibitory molecule CTLA-4 [43].

#### 1.1.2.3.1 Tolerogenic dendritic cells

Although DCs were originally recognized as the most potent stimulators of adaptive immunity, DCs play an important role in regulating immunity. These so called tolerogenic DCs regulate immunity by their ability to induce and maintain immune tolerance.

Tolerogenic DCs play for example fundamental role in negative selection and induction of Tregs in the thymus [44]. DCs with tolerogenic functions are also critical in maintaining peripheral tolerance by producing low levels of pro-inflammatory cytokines, expressing higher ratio of immunoinhibitory to co-stimulatory molecules and through the generation of anergic and regulatory T cells [45]. The tolerogenic properties of DCs can depend on their maturation state. During steady state, immature suppressing DCs are believed to continuously present self-antigens to circulating T cells [46]. This mechanism can be manipulated and several reports demonstrate that exposure to anti-inflammatory and immunosuppressive agents can condition DCs to a tolerogenic state [47-49].

## 1.2 ATHEROSCLEROSIS – AN INFLAMMATORY DISEASE

Atherosclerosis is a slow progressive chronic disorder of large and medium-sized arteries, and the main underlying pathological process of cardiovascular diseases (CVD), such as coronary artery disease (CAD) and cerebrovascular disease [50]. 16.7 million people are believed to die from CVD each year, making it the leading cause of mortality worldwide [51, 52].

Observational data support a strong association between plasma lipid levels and the risk of CVD, and for many years it was believed that atherosclerosis was merely a passive accumulation of cholesterol in the vessel wall. Today, the picture is much more complex, with atherosclerosis being thought of as a chronic inflammatory disease [50].

Genetically modified animals are helpful for dissecting pathological processes, and the most commonly used for atherosclerosis research are the hypercholesterolemic mice deficient in either the apolipoprotein E (*ApoE*<sup>-/-</sup>) or the LDL receptor (*Ldlr*<sup>-/-</sup>). Experiments in animal models as well as clinical and histopathological studies of patients groups have identified inflammatory mechanisms as being pathogenetically important in atherosclerosis. Components of both innate and adaptive immunity are involved in the disease process [50].

Classical epidemiology has established that hypertension, high plasma levels of LDL, cigarette smoking and diabetes are the most important risk factors for CVD [51, 53]. However, several biomarkers of inflammation have been suggested to carry a predictive value for CVD, such as CRP [54], IL-6 [55, 56], lipoprotein-associated phospholipase A2 and matrix metalloproteinase (MMP)-9 [56]. Treatment of individuals having high plasma values of CRP but no hyperlipidemia, with a lipid-lowering statin significantly reduced major cardiovascular events such as myocardial infarction (MI) [54]. This study showed that CRP measurements could identify individuals who are not eligible for therapy according to traditional approaches but who could benefit from treatment with a statin that has potent LDL-lowering and anti-inflammatory effects [57].

Further examples come from small and medium-sized genetic association studies which show that several inflammatory or immune-related genes contribute to CAD, such as genes for the cytokine IL-6 [58], OX40L important for T cell activation [59], enzymes involved in the biosynthesis of inflammatory leukotrienes [60-62], and the MCH II transactivator important for antigen presentation to T cells [63]. Furthermore, a recent report illustrated that variants in the chromosome 9p21 region, shown to be associated with risk of MI in genome-wide association studies, impaired IFN $\gamma$  signaling [64].

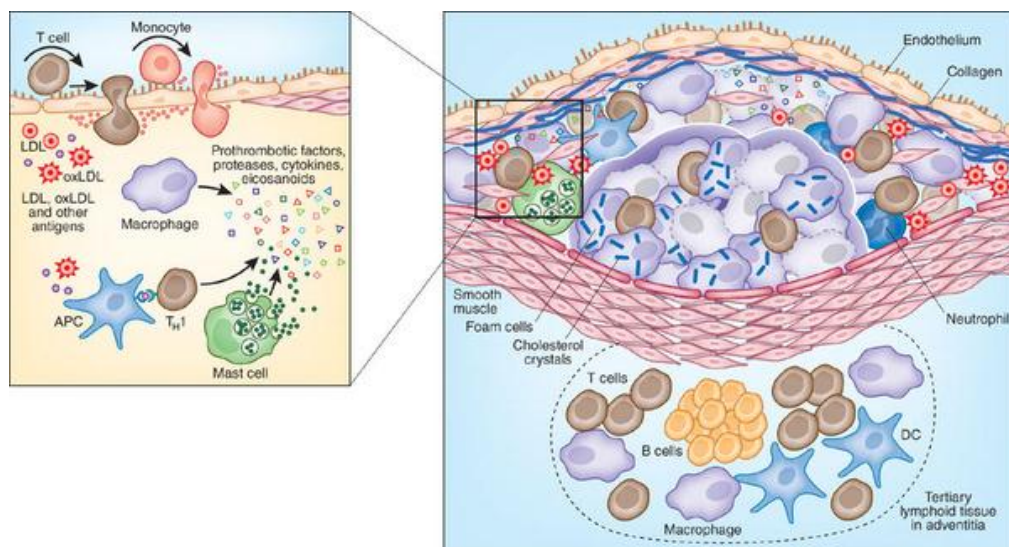
In addition, studies showing more cardiovascular morbidity in patients with chronic inflammatory disease, such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and psoriasis, point to a disease-promoting role for systemic inflammation in atherosclerosis [65-68].

### 1.2.1 Pathogenesis of atherosclerosis

The artery wall is composed of three layers. The inner layer, the tunica intima, is lined by a monolayer of endothelial cells (ECs) that is in contact with blood overlying a basement membrane. The middle layer, tunica media, contains SMCs embedded in a complex ECM. The outer layer, tunica adventitia, contains immune cells, nerve endings and microvessels [57, 69].

Atherogenesis is the formation of a plaque or lesion in the intimal layer of the vessel wall (*Figure 1*), and can be divided into several stages. The first macroscopically identifiable atherosclerotic alteration of the arterial is depositions of lipids in the intima called fatty streaks. These early lesions can in some cases even be found in the arteries of small children [70].

Cholesterol is transported in the blood mainly by LDL. These particles contain esterified cholesterol and triglycerides surrounded by a shell of phospholipids, free cholesterol and the high-molecular-weight apolipoprotein B100 (ApoB100). Circulating LDL particles can accumulate in the vessel wall, where ApoB100 binds to proteoglycans of the ECM through ionic interactions [71-73]. This is an important initiating factor in early atherogenesis [74].



**Figure 1.** The atheroma has a core of lipids, including cholesterol crystals, living and apoptotic cells and a fibrous cap with smooth muscle cells and collagen. Plasma lipoproteins accumulate in the subendothelial region. Several types of cells of the immune response are present throughout the atheroma including macrophages, T cells, mast cells and DCs. The atheroma builds up in the intima, the innermost layer of the artery. Outside the intima, the media contains smooth muscle cells that regulate blood pressure and regional perfusion, and further abluminally, the adventitia continues into the surrounding connective tissue. Here, cells of the immune response accumulate outside advanced atheroma and may develop into tertiary lymphoid structures with germinal centers. APC, antigen-presenting cell. *Reprinted by permission from Macmillan Publishers Ltd: Nature Immunology, Hansson et al. 2011 Mar;12(3):204-12, Copyright 2011.*

As a consequence of this subendothelial retention, LDL particles are trapped in the intima, where they are prone to oxidative modifications caused by enzymatic attacks or by reactive oxygen species [75, 76]. The modifications generate pro-inflammatory components such as truncated lipids, bioactive peptides and molecular species, e.g. malondialdehyde (MDA) adducts and 4-hydroxynonenal, forming on lysyl residues of ApoB100 [77-79]. Modified LDL components activate ECs and macrophages to produce adhesion molecules (E-selectin, VCAM-1) and chemokines (CCL2, CCL5, CXCL10, CX3CL1, SCF) [80-85].

Leukocytes, such as monocytes and T cells that adhere to the endothelium are induced by chemokines, e.g. CCL2, CXCL10, and SCF, to migrate into the intima. Oxidation of LDL increases binding to ScRs, especially SR-A1 and CD36, which results in endocytosis by monocyte-derived macrophages [86, 87]. The ensuing cholesterol accumulation eventually turns these macrophages into foam cells. DCs that patrol arteries may also take up LDL components for subsequent antigen presentation in regional lymph nodes.

With time a more complex plaque develops, with apoptotic as well as necrotic cells and cholesterol crystals forming a necrotic core of the lesion. The core is covered by a fibrous cap of variable thickness and its shoulder region are infiltrated by activated T cells, macrophages, mast cells, DCs and NKT cells which produce pro-inflammatory mediators and enzymes [88-93].

Plaques generally cause clinical manifestations by producing flow-limiting stenosis (narrowing of the lumen) that lead to tissue ischemia, or by rupture which exposes pro-coagulant material in the plaque's core to coagulant proteins in the blood, triggering thrombosis [94]. Plaques that rupture typically have thin, collagen-poor fibrous caps with few SMCs but abundant in macrophages. The inflammatory cells induce plaque disruption by production of collagenolytic enzymes that can both degrade collagen and generate mediators that provoke death of the SMCs, the source of arterial collagen. A thrombus can either interrupt blood flow locally or detach to become an embolus that can block blood flow in distal arteries [57].

### **1.2.2 Innate immunity in atherosclerosis**

Innate immune responses have a major role in the initiation of atherosclerosis [95]. Monocytes/macrophages, the major cell type in atherosclerotic lesions, participate in all stages of plaque development [83]. Their ScRs serve major roles as mediators of intracellular cholesterol accumulation, however, gene-knockout studies have provided contradictory results on their *in vivo* role in atherogenesis [96]. Many cell types in the plaque express TLRs (TLR1, TLR2, TLR3, TLR4, TLR5, TLR7 and TLR9), and become activated when binding PAMPs and/or DAMPs [97, 98]. TLRs are believed to be pro-atherogenic, however, recent studies have also shown disease-attenuating effects by certain TLR family members [98, 99].

In the context of atherosclerosis, the most interesting DAMP is probably oxLDL. LDL that is minimally modified by oxidation induces TLR4 and TLR2 activation in macrophages [100-104] and this detection of oxLDL appears to be mediated in

combination with ScRs such as CD36 [105]. Given that oxLDL is a complex structure that contains a mixture of possible ligands, including apolipoproteins, cholesterol, and phospholipids, it is difficult to determine the exact ligand that mediates receptor binding.

Mast cells are also important in the atherosclerotic process and these cells are believed to contribute to local inflammation by releasing proteases, lipid mediators and cytokines [83, 84, 106, 107]. Mast cells found in atherosclerotic plaques are mostly connective-tissue-type mast cells and are generally chymase and tryptase positive [108, 109].

Even though NK cells and NKT cells belong to the less common cell populations in the plaque, they are important producer of pro-inflammatory cytokines during atherogenesis [110-113]. Other important innate components of atherosclerosis are the complement system, from which certain member proteins have been found in the lesions [114]. Data from experimental models suggest a dual role for the complement system in atherosclerosis development [115, 116]. Recent reports also indicate a role for neutrophils in atherogenesis [117]. DCs are also present in the plaque where they most likely take part in presenting antigens to T cells, thereby activating the adaptive arm of the immune system [118].

#### *1.2.2.1 Dendritic cells in atherosclerosis*

DCs are key initiators and regulators of immune processes in atherosclerosis [119]. In the wall of healthy arteries, DCs reside in the intima and the adventitia where they survey the blood and surrounding tissue for danger signals [120-122]. DCs are more frequent in the plaque than in healthy vessel wall and may migrate to draining lymph nodes to present antigen to T cells and initiate a response of the adaptive immune system [119, 123]. However, studies show that emigration of DCs through nearby lymphatic vessels is impaired in atherosclerosis [124]. T cell activation might also occur within the atherosclerotic plaque as clustering of DCs and T cells has been observed [118, 120]. Depletion of the DC chemokine receptor CX3CR1, or the myeloid DC marker CD11c resulted in reduced atherosclerosis [125, 126]. In contrast, depletion of pDC accelerated lesion development in *Ldlr*<sup>-/-</sup> mice [127]. These and other findings highlight a role for DCs in the disease development.

### **1.2.3 Adaptive immunity in atherosclerosis**

Components of adaptive immunity are present in lesions throughout the course of atherosclerosis, and antigen-specific adaptive immune responses most likely play an important role in the atherogenic process [50, 128]. When *Rag1*<sup>-/-</sup> or *Scid/Scid* mice, two different models lacking functional T and B cells, were crossed with atherosclerosis prone *Apoe*<sup>-/-</sup> or *Ldlr*<sup>-/-</sup> mice, early atherosclerotic lesions formation was significantly reduced. [129, 130] [131, 132]. Furthermore, a large body of evidence suggests that both humoral and cellular immunity have a role in lesion development.



### 1.2.3.1 B cells in atherosclerosis

Atherosclerosis is associated with B cell activation [50, 133]. A low number of B cells are found in atherosclerotic plaques of mice and humans. These cells seem to be localized preferentially in the adventitia of the aorta where they have been described to form small lymphoid follicles [134-138]. Antibodies, both IgM and IgG, are also present within atherosclerotic plaques at all stages of lesions development [134, 139].

Several studies support a protective role of humoral immune responses in the disease development. Splenectomy accelerates atherosclerotic lesion formation, whereas transfer of splenic B cells from aged atherosclerotic *Apoe*<sup>-/-</sup> mice reduced disease development in splenectomized recipients [140]. Bone marrow transfer from B cell deficient *μMT* mice into *Ldlr*<sup>-/-</sup> mice showed that B cells and/or antibodies are protective in both early and late atherosclerosis [141]. A marked decrease in the number of B cells following interference with B-1 cell IgM production accelerated atherosclerotic lesion formation [142].

Recent reports demonstrate that different B cell subpopulations exert opposing effects on disease. B cell depletion by anti-CD20 antibody treatment was atheroprotective, and whereas transfer of B-2 cells aggravated disease, injection of B-1a cells was atheroprotective [142-144].

### 1.2.3.2 T cells in atherosclerosis

T cells of the atherosclerotic plaque are of the memory-effector phenotype and are mostly positive for TCRαβ and CD4 [145]. Clonal expansion of T cells has been demonstrated in lesions from both human and *Apoe*<sup>-/-</sup> mice, suggesting that antigen-specific reactions are taking place [146, 147]. Animal studies suggest that the T-cell response is pro-atherogenic. Absence of CD4<sup>+</sup> Th cells leads to reduced atherosclerosis, while transfer of the same cell type into *Scid/Scid/Apoe*<sup>-/-</sup> mice accelerated lesion development, with homing of T cells to the lesions [131, 148, 149].

Most of the CD4<sup>+</sup> T cells in lesions are of the Th1 type, and the signature Th1 cytokine IFNγ is also present [145]. IFNγ has long been implicated in atherogenesis and its pro-atherogenic effects are many including; reduction in collagen fiber formation, increased expression of MHC II, enhanced protease and chemokine secretion, upregulation of adhesion molecules, induction of pro-inflammatory cytokines, and enhanced activation of macrophages and ECs [145]. Mouse studies using either gene-knockouts or cytokine treatments to study one of the subsequent molecules; IFNγ and its receptor, the Th1-promoting cytokines IL-12 and IL-18, or the Th1-differentiating transcription factor T-bet, have shown pro-atherogenic effects of Th1 responses [150-159].

Studies on other Th cell subsets have generated conflicting data and further studies are required to fully understand their function in atherogenesis. Th2 cells, known to secrete IL-4, IL-5, IL-10 and IL-13, are predicted to be protective against atherosclerosis, but their role is not completely clear. More recently, the role of Th17 cells has been evaluated, showing both pro- and anti-atherogenic roles [160]. Furthermore, CD8<sup>+</sup> T cells can also be found in lesions and are believed to play a pathogenic role in the disease [160-164].



Clearer roles have been described for various subsets of Tregs in models of atherosclerosis, where several studies have demonstrated a protective effect of these cells. Antibody depletion of Tregs significantly increases atherosclerotic plaque size, and transfer of natural FoxP3<sup>+</sup> Tregs is protective [165, 166]. In addition, IL-10 or TGFβ, the two cytokines responsible for most of the effects mediated by Tregs, have been shown to have potent anti-atherogenic activities [167-171].

#### 1.2.4 Antigens in atherosclerosis

The clonal expansion of T cells and their clustering in close proximity to DCs and macrophages point to a local immune response in the plaque [50]. Several candidate antigens have been linked to the disease. Some of them are microbial molecules derived from both bacterial pathogens, such as *Chlamydomphila pneumonia*, and viral pathogens, such as cytomegalovirus [172, 173].

Although studies have implicated the involvement of infectious microorganisms in the atherosclerotic process, a large body of evidence suggests that self-antigens are the major target, and atherosclerosis can to some extent be seen as an autoimmune disease [174]. Acute manifestations of atherosclerotic disease does not show the same association with a restricted number of human leukocyte antigen types (MHC) as type I diabetes and rheumatoid arthritis, suggesting the involvement of multiple rather than single autoantigens [175]. However, the two autoantigens pointed out to be the most important are heat-shock protein 60 (hsp60) and LDL [50].

##### 1.2.4.1 Low density lipoprotein – an autoantigen

LDL is the antigen that has gained most attention in the atherosclerotic process. LDL contains both B cell and T cell epitopes, thereby eliciting both cellular and humoral responses during the course of atherosclerosis.

The activated humoral immunity against LDL results in formation of antibodies that recognize MDA-lysine and other oxidatively generated epitopes of LDL particles. These autoantibodies can be found in circulation of humans and experimental animals [176, 177] [178, 179]. B-1 B cells produce natural IgM antibodies against oxidized phospholipids, present not only on oxLDL but also in the cell wall of *Streptococcus pneumonia* and on apoptotic cells [180]. Antibodies to native and MDA-modified ApoB100 are mostly of the IgG isotype [181]. Screening of a peptide library resulted in binding of autoantibodies to more than 100 different ApoB100 peptides [182]. Experimental studies in mice suggest that oxLDL-autoantibodies, especially of the IgM isotype, are atheroprotective [183, 184]. However, their association with atherosclerosis and CVD remains unclear [185, 186].

The presence of IgG antibodies recognizing LDL and components thereof implies that isotype switching must have been activated by T cells specific for the same antigen. There is today clear evidence that LDL-reactive T cells exist in circulation and lymphoid organs, as well as in atherosclerotic plaques [187, 188]. Several reports propose that they are detrimental; adoptive transfer of CD4<sup>+</sup> T cells from MDA-LDL immunized mice accelerated atherosclerosis in *Scid/Scid/ApoE<sup>-/-</sup>* mice while *in vivo*

blocking of ApoB100 reactive T cells reduced plaque burden [189, 190]. Recently, it was shown that these autoreactive T cells recognize peptide motifs of native LDL particles and ApoB100, and that oxidation extinguishes rather than promotes LDL-dependent T cell activation [189].

### **1.2.5 Immunomodulation as a treatment for CVD**

The treatment of atherosclerosis is currently based on lipid lowering by statins – inhibitors of hydroxymethyl glutaryl coenzyme A (HMG-CoA) reductase, involved in cholesterol metabolism. However, even in patients treated with statins a considerable residual burden of cardiovascular disease remains [57]. Besides effective LDL lowering, statin treatment reduces CRP levels in humans, and several studies suggest that some of their clinical benefits accrues from an anti-inflammatory action [191, 192]. This finding, together with discoveries of inflammatory pathways in atherosclerosis development may pave the way for immunomodulation as a new potential therapeutic approach against atherosclerosis. However, no existing systemic anti-inflammatory strategy, such as glucocorticoids, non-steroidal and anti-inflammatory drugs, or anti-cytokine agents has been proven as an ideal candidate for atherosclerosis treatment [57]. Many of them create unwanted side-effects, such as dyslipidemia, hypertension, and diabetes [193] [57]. Since it is now evident that atherosclerosis involves autoimmune reactions against LDL particles accumulating in the artery wall, new clinical applications targeting this aberrant antigen specific response would be appealing.

A very effective strategy in other disease pathologies is vaccination. Modern vaccines are cheap, highly specific, and have generally few adverse effects. A vast amount of experiments in animal models shows protective effects of vaccination with LDL or components thereof [194-199]. The underlying mechanism is not fully understood, but suggests effects on cholesterol levels and induction of protective antibodies [183, 184]. Consistent with this, injection of antibodies recognizing modified LDL components have resulted in reduced plaque burden in mouse models of atherosclerosis [200-202].

LDL is, however, a complex particle with an antigen composition that is difficult to standardize and it may potentially also contain harmful components. The use of ApoB100 peptides would be one way to bypass this problem, but the recent identification of an ApoB100 peptide able to trigger pro-atherogenic responses [85], emphasizes the importance in characterizing the precise epitopes involved in the induction of atheroprotective immunity

An alternative mechanism in atheroprotection by LDL immunization is alteration of the balance between pro-inflammatory and anti-inflammatory T cell subtypes [203, 204]. A frequently used method to induce antigen-specific Tregs is mucosal immunization.

Induction of LDL-specific Tregs was achieved by mucosal administration of a fusion protein with a peptide sequence of native LDL, and paralleled with decelerated atherosclerosis development [205]. Unfortunately, experience from other autoimmune diseases points to difficulties in developing mucosal tolerance-based immunotherapy for humans. Antigen-specific Tregs can also be induced by DCs made tolerogenic in

the presence of anti-inflammatory cytokines, and several reports show amelioration of autoimmune disease when such DCs were loaded with disease associated antigens [206-209]. Therefore, DC-based therapy has the potential of being an attractive option for treating atherosclerosis.

A potential disadvantage when using approaches to trigger T cell specific responses is that it may turn out to be necessary to perform HLA genotyping of patients before treatment since usage of T cell epitopes, such as ApoB100 peptides, may need to be individualized depending on HLA type. Nonetheless, applying treatment strategies directed against antigen-specific mechanisms remains attractive. A vaccine candidate (CVX-210H), based on an ApoB100 peptide, has already been developed [182], and an antibody against MDA-modified ApoB100 (BI-204) is currently in phase II of clinical development [210].

## 2 AIMS

The studies included in this thesis have investigated the role of different immune cells, as well as proteases they secrete, in the pathological process of atherosclerosis. More specifically, the aims were to;

- Determine the role of immunogenic dendritic cells presenting modified LDL in the development of atherosclerosis.
- Assess the role of tolerogenic dendritic cells presenting apolipoprotein B100 in the development of atherosclerosis.
- Determine the role of adaptive immunity in immunization-induced atheroprotection.
- Evaluate the role of serine protease inhibitor A3 (serpinA3) in atherosclerosis and aneurysm formation.

## 3 METHODOLOGICAL CONSIDERATIONS

### 3.1 MURINE MODELS OF ATHEROSCLEROSIS

Murine models constitute the most widely used experimental system to study atherosclerosis and their main advantages include reproducibility and the availability of transgenic technology to dissect the relevant pathologic processes. Generally, mice are resistant to atherogenesis. So far, very few strains are known to develop atherogenesis after feeding with an atherogenic diet. One of these strains is the C57Bl/6 mouse, which is the standard model for atherosclerosis research, and is the background of all mouse models used in this thesis. However, the C57Bl/6 strain only develops small fatty streaks, and it was not until the production of several gene knockouts and transgenic mice became available that the wide spread use of mouse models in atherosclerosis research started. The genetically modified mice displayed remarkable effects on plasma lipoproteins and could develop larger and more complex lesions. Thus far, 239 strains for atherosclerosis research have been published on the Jax Laboratory webpage. Among them, mice deficient in apolipoprotein E (*Apoe*<sup>-/-</sup>), LDL receptor (*Ldlr*<sup>-/-</sup>), and human apoB100 transgenic mice (huB100<sup>tg</sup>) display marked atherogenesis throughout their arterial tree [211, 212].

However, limitations of genetically modified mouse models should be kept in mind when translating findings in mouse studies to human atherosclerosis. The mouse immune system diverges in many ways from that of humans, and the cholesterol levels required for atherogenesis in mice exceed those encountered in the clinic, and does not reflect the chronic nature or complexity to the human disease [57]. Most importantly, plaque rupture with thrombosis – one of the main causes of clinical manifestations by atherosclerosis – seldom develop in experimental mouse models [57].

#### 3.1.1 *Apoe*<sup>-/-</sup> mice (paper I, III, IV)

The mouse *Apoe* gene was the first mouse gene successfully deleted for atherosclerosis research [213, 214]. ApoE is the ligand for clearance of remnant lipoproteins by the liver and the *Apoe*<sup>-/-</sup> mouse develops severe hypercholesterolemia. These mice spontaneously develop atherosclerosis on a normal chow diet (low fat and low cholesterol diet) and at 20 weeks of age display fibrous plaques similar to human atherosclerosis, both in phenotype and distribution [215].

Disadvantages with this model are that the lipoprotein profile is dominated by elevated VLDL, whereas humans carry most their cholesterol in the LDL fraction, and the involvement of ApoE in other processes such as immune activation and proliferation of stem cells in the bone marrow is also lost [216, 217].

#### 3.1.2 *μMT/Apoe*<sup>-/-</sup> mice (paper III)

*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. By crossing the *μMT* mouse with an *Apoe*<sup>-/-</sup> mouse you get an atherosclerotic mouse model completely deficient in mature B cells and antibodies. The *μMT/Apoe*<sup>-/-</sup> mouse used in paper III were provided Drs Francis Bayard and Rima Elhage in Toulouse, France[218].

### 3.1.3 Rag-2<sup>-/-</sup>/ApoE<sup>-/-</sup> mice (paper III)

Recombinase activator gene 2 (Rag-2) is necessary for lymphocyte-specific V(D)J recombination in normal developing lymphocytes, and Rag-2 deficiency results in lack of functional T, as well as B cells [219]. The ApoE<sup>-/-</sup> and RAG-2-(B6-Rag2<sup>tm1</sup>) deficient mice (Rag2<sup>-/-</sup>/ApoE<sup>-/-</sup>) used in paper III was backcrossed into a C57BL/6J background for six generations [220].

### 3.1.4 huB100<sup>tg</sup>xLdlr<sup>-/-</sup> mice (paper II)

*Ldlr*<sup>-/-</sup> mice do not develop significant atherosclerotic lesions on a normal chow diet [221]. However, in response to a high fat diet, they develop more severe hypercholesterolemia and robust atherosclerotic lesions throughout the aortic tree [222-224]. Mice transgenic for human ApoB100 show humanized lipoprotein profiles and enables studies on cellular immune responses to human LDL-derived epitopes [225]. Just as the *Ldlr*<sup>-/-</sup> mice, these mice require a high fat diet for atherosclerosis development [226]. In paper II we used huB100<sup>tg</sup>x*Ldlr*<sup>-/-</sup> mice, a cross between huB100<sup>tg</sup> and *Ldlr*<sup>-/-</sup> mice, with an additional mutation in the *APOB100* gene that hinders the ApoB100 mRNA editing into ApoB48, a process that occurs in mouse but not human liver [74]. This mouse strain develops atherosclerosis spontaneously on a chow diet [227], but when fed high fat diet this results in accelerated atherosclerosis development.

### 3.1.5 SerpinA3n transgenic mice (paper IV)

To study the role of serine protease inhibitor A3 (serpinA3) in atherosclerosis and aneurysm disease, we generated transgenic mice. In mice, the *serpinA3* gene has undergone extensive duplication and diversification resulting in a family of 13 closely related inhibitors with differing tissue distribution and protease specificity [228, 229]. Gene expression and functional studies suggest that serpinA3n is the closest murine orthologue of human serpinA3 [229, 230].

The murine serpinA3n cDNA sequence was amplified from C57Bl/6 mice and cloned into the plasmid chicken beta-actin promoter plasmid pCAGIPuro, in which serpinA3n expression is driven by a human cytomegalovirus immediate cytomegalovirus early enhancer (HCMVIEE) coupled to the chicken beta-actin promoter. The sequence was confirmed by sequencing. Using DNA microinjection into C57Bl/6 fertilized oocytes of the beta-actin promoter-HCMVIEE-*serpinA3n* fragment, we obtained two founder mice positive for the *serpinA3n* transgene. The SerpinA3n transgenic mice were identified by PCR. Functional overexpression of serpinA3n was confirmed by measurements, in plasma and aorta, of serpinA3n gene and protein, as well as activity of the enzymes it is known to inhibit. Both transgenic strains were used to study aneurysm formation, and one of the serpinA3n transgenic mouse strains was crossed with *ApoE*<sup>-/-</sup> mice to study atherosclerotic lesion development.

## 3.2 LDL PREPARATIONS (PAPERS I-III)

In experiments such as immunization or *in vitro* culture of cells where LDL was used, it was always freshly prepared either from mouse or human plasma. Murine LDL obtained from *ApoE*<sup>-/-</sup> mice was used in paper I and paper III to reduce the risk for inter-species interactions. LDL was isolated from plasma by ultracentrifugation using either

a one-step or a two-step method. In the former technique the plasma was centrifuged through a discontinuous NaCl gradient for 20 h with subsequent collection of the fraction between 1.020 mg/ml and 1.063 mg/ml. This density cutoff should contain mainly LDL particles. In the two-step centrifugation method plasma is first centrifuged 12 h through 1.020 mg/ml of KBr and then in a second round through 1.063 mg/ml [231]. After the first round chylomicrons and VLDL are removed from the top, and after the second round the LDL particles are removed from the top. In preparations of native LDL, 1 mM EDTA was always present in preparations, and protease inhibitors were added in the plasma for all preparations used. The purified LDL was used for MDA-modification or isolation of ApoB100.

### **3.2.1 MDA-modification of LDL (paper I and III)**

MDA-modification results in adducts that are known to be present in oxLDL *in vivo* [232, 233]. MDA was produced by acid hydrolysis of malondialdehyde-bis-dimethylacetal, and LDL was incubated with 0.5 M MDA for 3 h at 37°C 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 ApoB preparation (paper II)**

ApoB100 is not a soluble protein and needs certain measures to stay soluble after delipidation. We used a standard chloroform/methanol/water extraction protocol to remove lipids from LDL. The insoluble extracted ApoB100 was then resuspended in a minimal volume of 10% SDS until it dissolved completely. This unclean preparation was run on a PD-10 desalting column to remove excess SDS, and then purified on a Superdex 200 size-exclusion column using Tris-HCl buffer. The purified ApoB100 was always tested in cell cultures to see to control for toxicity due to remaining SDS in the preparation. It is reasonable to believe that some SDS will remain bound to the protein in order to keep it solubilized in salt solutions. However, the ApoB100 preparations were more than 90% pure and kept at pH 7.4.

## **3.3 T CELL ASSAYS (PAPERS I-III)**

Most of our T cell studies focused on proliferation of antigen-specific T cells. We either used total splenocytes, or purified T cells together with irradiated splenocytes as APC, in the presence of antigen. In our experience it is better to use a coherent system such as total splenocytes, rather than purified T cells. The usage of splenocytes is a more *in vivo*-like system and less vulnerable compared to cumbersome purification of specific cell types. It is also easy to analyze specific populations of spleen in the flow cytometer when needed. However in some cases (paper I and III) where CD4<sup>+</sup> T cells were absolutely needed, purification was performed. In this case a commercially available column was used that negatively selected T cells in order to minimize activation of the T cells. Proliferation was measured by <sup>3</sup>H-Thymidine incorporation as detected by a  $\beta$ -counter. In some instances we also measured IL-2 to estimate T cell activation, or other cytokines that would suggest T effector differentiation such as IFN $\gamma$ , IL-10, IL-5 and others. Data were sometimes presented as stimulation index (SI) when baseline value varied between groups. SI is calculated by subtracting the baseline value from the stimulated value and dividing by the baseline value. In all T cell assays except those

with T hybridoma cells, we used serum-free medium supplemented with ITS (insulin, transferrin and selenium). This medium worked much better than regular fetal calf serum containing medium, since small amount of LDL in the latter medium caused high background proliferation.

### 3.4 GENERATION AND TREATMENT OF DC (PAPERS I AND II)

In order to generate high amount of murine DCs, bone marrow was cultured in the presence of GM-CSF and IL-4. This cytokine environment will also induce differentiation of other myeloid cells including granulocytes and macrophages [234]. For this reason we purified DC by density centrifugation in Optiprep (commercially available sugar solution). More than 70% of the resulting fraction of DCs expressed the surface markers CD11c, I-A<sup>b</sup> and co-stimulatory molecules, indicating that many cells were in a mature state. They could however take up antigen and were effective in presentation to T cells. This was not the case for the cells that remained unselected after density gradient purification. Antigens were added to DCs and incubated overnight together with LPS, which was used to induce complete maturation and to avoid antigen specific maturation effects. In paper II we reduced the concentration of LPS by titration to lowest possible, yet able to trigger maturation. We also changed the purification protocol from density gradient to CD11c purification column. These measures allowed us to receive more than 95% pure DCs that contained a mix of immature cells and mature cells that were slightly pushed into maturation. It was necessary not to have highly matured DCs during treatment with IL-10 in the antigen pulse. Consequently, during antigen pulse we waited a few hours before adding the low LPS concentration, which also allowed us to have more efficient uptake by immature DC. Compared to the previous protocol the mature DCs were equally good presenters of antigen to T cells and expressed similar levels of co-stimulatory molecules. In paper I we used MDA-LDL as antigen and in paper II we prepared the pure protein ApoB100 for antigen pulsing of DCs. This together with the changed procedure for DC purification and maturation will render comparisons in treatment results somewhat difficult and must be kept in mind when interpreting data from papers I and II.

### 3.5 IMMUNIZATIONS (PAPER III)

For immunizations, 100 µg MDA-LDL, emulsified in Freund's adjuvant at a volume ratio of 1:1, was subcutaneously injected per mouse. MDA-LDL was emulsified in complete Freund's adjuvant (CFA) for the first injection and in incomplete Freund's adjuvant (IFA) for booster injections. Since Freund's adjuvant itself has been shown to have effects on atherogenesis [148, 180, 235], one control group was treated with adjuvant alone, and one group of mice remained untreated.  $\mu MT/ApoE^{-/-}$  mice were injected with a two-week interval, which has been shown to be protective in both  $ApoE^{-/-}$  and  $CD4^{-/-}/ApoE^{-/-}$  mice [148, 198].  $Rag2^{-/-}/ApoE^{-/-}$  mice were instead injected with a four-week interval. The prolonged time interval was necessary due to new ethical rules for animal experiments issued by Jordbruksverket, the governmental board supervising animal experiments in Sweden, but despite this the atheroprotective effect in  $ApoE^{-/-}$  mice was retained.



## 4 RESULTS AND DISCUSSION

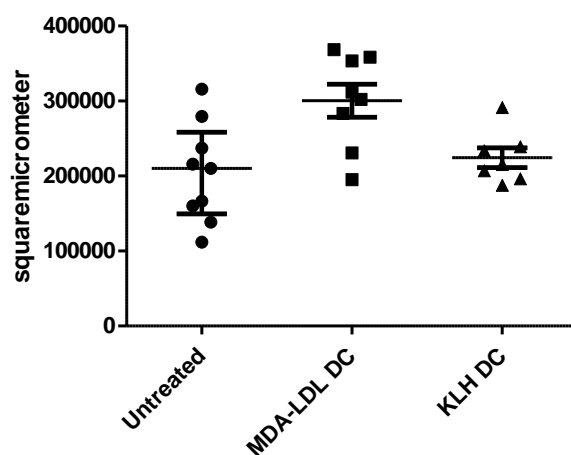
### 4.1 MDA-LDL PULSED DC AGGRAVATE ATHEROSCLEROSIS (PAPER I)

DCs are the most potent activators of naïve T cells [25]. DCs are present in atherosclerotic plaques, in which they take up antigens and present them to T cells, either in the draining lymph nodes or locally in the plaque [118]. One of the most important antigens in atherosclerosis is LDL, and LDL-reactive T cells are suggested to be deleterious for the disease [189, 190]. However, immunization of LDL or components thereof emulsified in adjuvant is atheroprotective [194-198].

To explore the role of DCs in atherosclerosis, we pulsed bone marrow-derived DCs with MDA-LDL, a model antigen of oxLDL, and injected these DCs subcutaneously into *Apoe*<sup>-/-</sup> mice. The effect on atherosclerosis development was compared to mice injected with DCs pulsed with the disease irrelevant antigen keyhole limpet hemocyanin (KLH), or to mice that remained untreated.

Mildly oxidized LDL is able to induce maturation of DC [236, 237]. Activation of DCs is also induced by ligation of a TLR ligand, such as LPS [238, 239]. To be able to study the effect of a disease-related antigen versus a disease irrelevant antigen on the outcome on atherosclerosis, and not due to a difference in DC maturation level, all DCs also received a high dose of LPS.

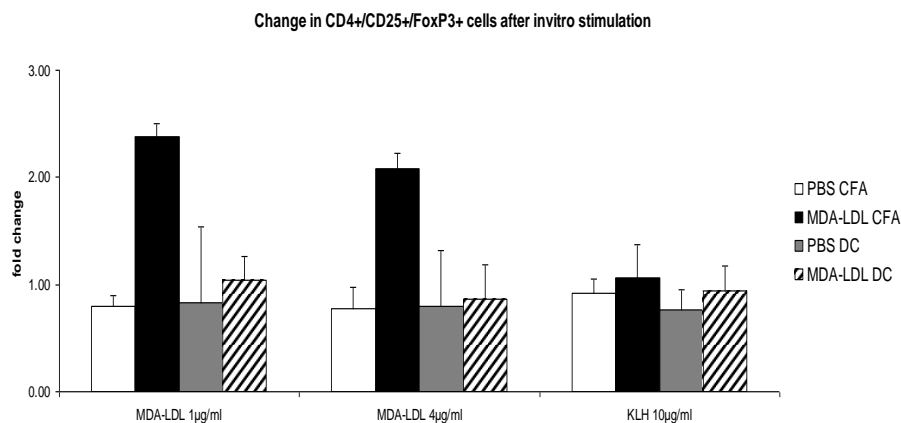
*In vivo* detection studies have shown that injected antigen-bearing DCs migrate to draining lymph nodes where they interact with antigen-specific T cells [240]. In our study the induction of IL-6 in the inguinal lymph nodes of DC-immunized mice suggested that the treatment initiated an immune response in the draining lymph nodes. Mice that receive antigen-pulsed DCs show increased T cell responses to the cognate antigen [240]. In line with this, T cells purified from draining lymph nodes of mice treated with MDA-LDL or KLH loaded DCs, proliferated *in vitro* to MDA-LDL and KLH respectively. This response was accompanied by a burst of IFN $\gamma$  secretion and an induction of circulating antigen-specific antibodies.



**Figure 2.** Effects of pulsed DC-transfer on atherosclerosis lesion development. Morphometric quantitation of lesion size ( $\mu\text{m}^2$ ) in the aortic root of *Apoe*<sup>-/-</sup> mice.

Only immune induction by the disease-related antigen MDA-LDL affected the atherosclerotic process. Treatment with MDA-LDL pulsed DCs, but not with KLH pulsed DCs, worsened atherosclerotic disease since the mice exhibited significantly larger and more inflamed atherosclerotic lesions compared to untreated animals (*Figure 2*).

Our treatment with MDA-LDL pulsed DCs accelerates atherogenesis, while we and others have shown that parental immunization with modified LDL emulsified in adjuvant reduce atherosclerosis development. Remarkably, both treatment strategies results in increased antigen-specific T cell and antibody responses [194-198]. However, when comparing the two different regimens, we found that only MDA-LDL emulsified in CFA lead to induction of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs (*Figure 3*), which could explain the atheroprotective effects of this regimen and the lack of protection by MDA-DC pulsed DCs. Indeed, several reports have shown the atheroprotective potential of Tregs [165-171].



**Figure 3.** Proliferation of T cells *in vitro* in response to antigen. Fold induction in percentage of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T cells of total CD3<sup>+</sup> T cells.

This finding illustrates that repeated immunization with antigen-loaded immunogenic DCs increases atherosclerosis. In contrast, it was recently reported that immunogenic DCs loaded with oxLDL reduced lesions induced when a collar was surgically attached to the carotid artery of hypercholesterolemic mice [241]. These DCs were prepared in a different way compared to our protocol, as well as injected by another route in a different mouse model. The study also failed to show any differences in the aorta of treated mice, which suggests that the local environment determines the outcome.

In addition, our results showing detrimental properties of immunogenic DCs confirm and extend studies showing that immunogenic DCs loaded with an artificial antigen expressed transgenically in the artery wall accelerated atherosclerosis [164], and that an extended lifespan of DCs affected plaque inflammation and cholesterol metabolism [242].

## 4.2 TOLEROGENIC APOB100 PULSED DC ATTENUATES ATHEROSCLEROSIS (PAPER II)

In the normal artery, resident DCs are thought to maintain peripheral tolerance to self-antigens by silencing T cells [45] [50]. However, danger signals generated during atherogenesis may activate DC, leading to a switch from tolerance to the activation of adaptive immunity [50]. This could explain the existence of autoreactive LDL-specific T cells in humans and mouse models of atherosclerosis [189].

Tolerogenic DCs generate anergic and regulatory T cells by producing low levels of pro-inflammatory cytokines and by expressing higher ratio of coinhibitory to co-stimulatory molecules [45]. Several reports demonstrate that DCs can be conditioned into a tolerogenic state by exposure to immunosuppressive and anti-inflammatory agents, such as IL-10 [47-49, 243]. IL-10 has been shown to downregulate DC expression of MHC class II, co-stimulatory molecules such as CD80/CD86, chemokines, and pro-inflammatory cytokines, such as IL-6, IL-12 and TNF $\alpha$  [243]. This enables inhibition of both Th1 and Th2 immune responses by IL-10 treated DCs [243].

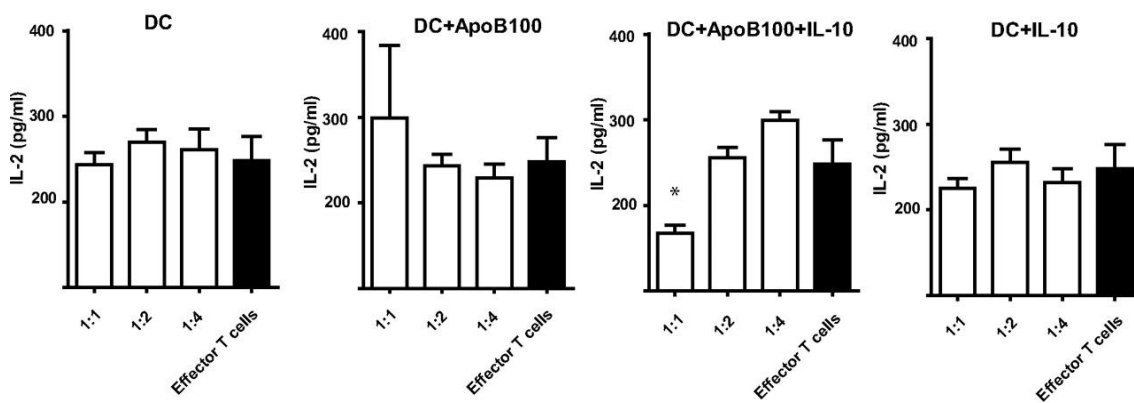
To investigate the role of tolerogenic DCs in atherosclerosis, we pulsed IL-10 stimulated bone marrow-derived DCs with ApoB100, the protein moiety of LDL, and injected these DCs intravenously into huB100<sup>tg</sup> $\times$ Ldlr<sup>-/-</sup> mice. One group of mice was left untreated, while the other control groups received injections of DC that were treated with IL-10 or ApoB100 alone, or kept in medium only. All DCs were also stimulated with the lowest possible LPS concentration needed for induction of maturation.

We hypothesized that by treating DCs with ApoB100 in the presence of IL-10 to create tolerogenic ApoB100-specific DC, we could modulate the aberrant cellular immune responses against LDL. Human ApoB100 was chosen as an antigen instead of oxLDL, since recent reports have shown that atherogenesis is driven by autoreactive T cells that recognize peptide motifs of native, rather than oxidized, LDL particles and ApoB100 [189]. The huB100<sup>tg</sup> $\times$ Ldlr<sup>-/-</sup> mouse model enabled the use of human ApoB100 as an antigen.

IL-10 treatment of bone marrow-derived DCs reduced their secretion of TNF $\alpha$  and CCL-2, and completely abrogated IL-12 production. However, no reduction in the expression of MHC class II or the co-stimulatory molecules CD80/CD86 was detected, presumably due to the co-administration of LPS to all DCs. Activated CD4<sup>+</sup> T cells that were incubated with such tolerogenic DCs significantly reduced their secretion of IFN $\gamma$  and upregulated the expression of PD-1 as compared to controls. PD-1 is a co-inhibitory molecule essential for the control of T cell activation and generation of FoxP3<sup>+</sup> Tregs [244]. Tolerogenic DC also induced a *de novo* generation of FoxP3<sup>+</sup>CD4<sup>+</sup> T cells. Interestingly, IL-10 induced tolerogenic DCs could suppress the proliferation of effector T cells, indicating induction of anergy. This is in line with previous observations [208, 245].

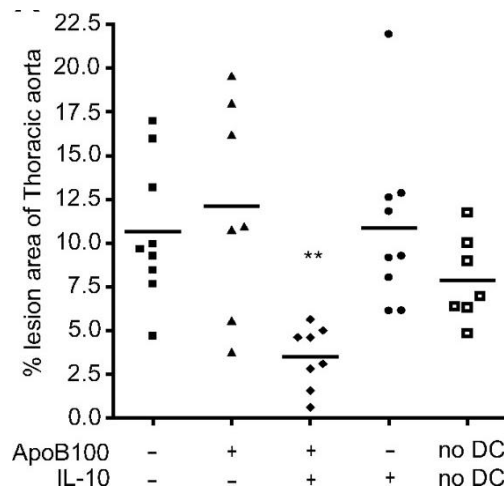
When tolerogenic DCs pulsed with ApoB100 were injected into huB100<sup>tg</sup> $\times$ Ldlr<sup>-/-</sup> mice, the splenic population of ApoB100-specific T cells became less prone to proliferate

against ApoB100. Th1 as well as Th2 responses were also decreased from *in vitro* re-challenged splenic T cells. Mice that received IL-10-treated DCs had significantly decreased plasma levels of IFN $\gamma$  and increased mRNA levels of IL-10, TGF $\beta$  and FoxP3 in the spleen, which shows that the DCs induced a regulatory and anti-inflammatory machinery. In line with this, we could demonstrate that CD4<sup>+</sup> T cells purified from spleens of mice treated with tolerogenic DCs pulsed with ApoB100, and not from any of the other groups, could suppress the activation of a T cell hybridoma that responds to ApoB100 (*Figure 4*). This indicates an antigen-dependent suppression of activation. In addition, *in vitro* presentation of ApoB100 by tolerogenic DCs significantly abolished the response of the ApoB100-specific T cell hybridoma.



**Figure 4.** Tolerogenic DCs generate antigen-specific suppressor regulatory T cells and downregulate antigen-specific T helper responses. DCs pulsed with or without ApoB100 and IL-10 were transferred intravenously to recipient mice. One week later,  $2 \times 10^4$  cells of the ApoB100-specific T hybridoma 48.5 were seeded together with  $1 \times 10^4$  DCs and 20  $\mu$ g/mL ApoB100 in 96-well plates. CD4<sup>+</sup> T cells purified from spleens of DC-treated mice were coincubated at different ratios with the T hybridoma effector cells. Antigen-specific T-cell activation was measured 24 hours later by interleukin-2 (IL-2) secretion in the supernatant.

Since IL-10 induced tolerogenic DCs modulated the cellular immunity to LDL-derived ApoB100 in such a way that peripheral tolerance to the antigen was increased, we hypothesized that their administration would slow down atherogenesis. Indeed, one single injection of tolerogenic ApoB100 pulsed DCs resulted in a significant, 70% reduction of lesions in the descending thoracic aorta, as compared to all other groups that received DCs, and a 50% reduction compared with untreated mice (*Figure 5*). Disease amelioration was paralleled by reduced CD4<sup>+</sup> T cell infiltration into lesions, suggesting that activation and recruitment of effector T cells into lesions are of major importance for the atheroprotective effect of tolerogenic DCs.



**Figure 5.** Tolerogenic DCs reduce atherosclerotic lesion formation in the thoracic aorta of huB100tg×Ldlr<sup>-/-</sup> mice. Mice received one intravenous injection with DCs loaded with or without ApoB100 and/or IL-10 and were fed a Western diet for 10 weeks.

Mice that received DCs pulsed with ApoB100 had a modest increase in atherosclerosis development. This is in line with the notion from our previous results (paper I) and by others, which show that immunogenic antigen-pulsed DCs aggravate inflammatory disease [164].

Antigen loaded DCs injected intravenously have also been shown to migrate to thymus, where they are involved in clonal deletion of developing antigen-specific T cells [246]. Maturation status influenced the ability of DCs to home to the thymus – stimulation with a high dose of LPS resulted in a smaller number of injected DCs in thymus [246]. One may presume that the decreased T cell responses against ApoB100 seen after transfer of ApoB100 pulsed tolerogenic DCs, may be due to increased central tolerance to ApoB100. It would be interesting to explore the effect on chemokine receptor expression and migration patterns of ApoB100-pulsed DCs stimulated with IL-10.

In conclusion, the present study show that tolerogenic DC therapy that targets immune reactions to the ApoB100 protein present in LDL, can attenuate systemic inflammation and significantly reduce atherosclerotic plaque burden.

#### 4.3 ADAPTIVE IMMUNITY IN ATHEROPROTECTION BY IMMUNIZATION (PAPER III)

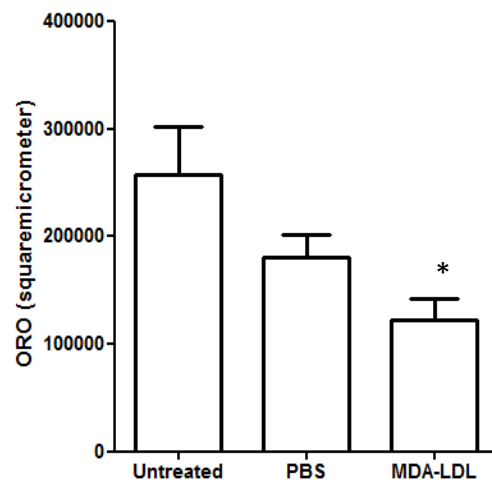
One of the first pieces of evidence that manipulating the immune system could be used as potential therapeutics for cardiovascular disease came from studies in which hypercholesterolemic rabbits were immunized with oxLDL emulsified in adjuvant. The conductors of that study were surprised when they found that oxLDL-vaccinated animals developed a partial protection against atherosclerosis. This observation has

subsequently been confirmed in a number of different animal models of atherosclerosis, including *Apoe*<sup>-/-</sup> mice [194-198].

The underlying mechanism of atheroprotection is, however, still not fully understood. LDL contains both B cell and T cell epitopes and protection is associated with activation of both humoral and cellular immunity. Atheroprotection after vaccination in mice correlates with titers of anti-oxLDL IgG antibodies, implying that antibody isotype switch is induced by activated antigen specific CD4<sup>+</sup> T cells [140, 247, 248]. Several experimental studies in mice suggest that oxLDL-autoantibodies are atheroprotective [183, 184]. Interestingly, immunization-induced atheroprotection still remains in the absence of CD4<sup>+</sup> T cells. Despite the absence of Th cells, increased levels of IgG antibodies specific for MDA-LDL were formed [148].

To explore the role of adaptive immunity in atheroprotection by oxLDL vaccination, different mouse models were used. *μMT*/*Apoe*<sup>-/-</sup> mice, lacking functional B cells and antibodies, were used to study the role of humoral immunity. *Rag2*<sup>-/-</sup>/*Apoe*<sup>-/-</sup> mice, which lack both functional T and B cells, were used to investigate whether adaptive immunity is critical for the protective effect.

The immunization protocol shown to be protective in *Apoe*<sup>-/-</sup> mice and *CD4*<sup>-/-</sup>/*Apoe*<sup>-/-</sup> mice [148, 198], was used to treat *μMT*/*Apoe*<sup>-/-</sup> mice. MDA-LDL was emulsified in Freund's adjuvant (CFA) for the first injection and in incomplete Freund's adjuvant (IFA) for booster injections. Since Freund's adjuvant itself has been shown to have effects on atherogenesis [148, 180, 235, 249], one control group was treated with adjuvant alone, and one group of mice remained untreated.

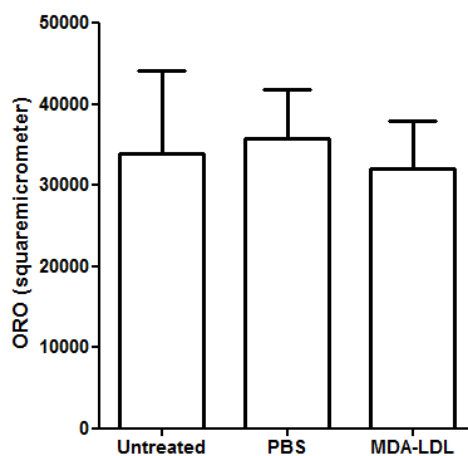


**Figure 6.** Lesion development in *μMT*/*Apoe*<sup>-/-</sup> mice. Morphometric quantitation of lesion size (μm<sup>2</sup>) in the aortic root.

We show that oxLDL vaccination resulted in reduced plaque burden in  $\mu MT/Apoe^{-/-}$  mice (Figure 6). This shows that oxLDL vaccination-induced atheroprotection is not dependent on B cells and antibodies. This is in line with another study where immunization with native LDL inhibited lesion development without inducing antibodies to oxLDL [250]. The treatment did not reduce local inflammation in the lesions, as there was no difference in lesional VCAM-1 expression between the groups.

Tregs are known to be atheroprotective [165, 166], and in paper I we showed that immunization with MDA-LDL in immunocompetent  $Apoe^{-/-}$  mice induces antigen-specific Tregs. However, to which degree cellular immunity is affected in absence of B cells is controversial - while some studies reveal a critical role for B cells in T cell responses [251, 252], others indicate that absence of B cells has little effect on T cell responsiveness [253-255]. We could detect proliferation of T cells from MDA-LDL immunized  $\mu MT/Apoe^{-/-}$  mice when these were re-challenged with the cognate antigen *in vitro*, although the responses was not as strong as those measured after immunization in  $Apoe^{-/-}$  mice. Importantly, induction of antigen-specific Tregs was seen after oxLDL vaccination in  $\mu MT/Apoe^{-/-}$  mice, suggesting that they may play a protective role also in this mouse model. The importance of Tregs for the atheroprotective effects of vaccination is supported by a recent study where the atheroprotective effect of immunization with an ApoB100-peptide in  $Apoe^{-/-}$  mice was abolished by administration of an antibody depleting Tregs [204].

We then wanted to investigate if oxLDL vaccination could elicit protection in complete absence of adaptive immunity. Due to new ethical rules by the governmental board supervising animal experiments in Sweden time intervals between immunizations had to be prolonged in  $Rag2^{-/-}/Apoe^{-/-}$  mice. To ensure that longer time intervals retained the protective effect, we immunized a new batch of  $Apoe^{-/-}$  mice and demonstrated that this immunization protocol still resulted in reduced atherosclerosis development with lowered lesional inflammation. In contrast, immunization of immunodeficient  $Rag2^{-/-}/Apoe^{-/-}$  mice with oxLDL had no effect on atherogenesis (Figure 7). This finding illustrates that complete lack of adaptive immunity abolishes the protective effect.



**Figure 7.** Lesion development in  $Rag2^{-/-}/ApoE^{-/-}$  mice. Morphometric quantitation of lesion size ( $\mu m^2$ ) in the aortic root.

The mechanism of vaccination against oxLDL is complex. Although protection remained in the absence of CD4<sup>+</sup> T cells or B cells, we could demonstrate that it is dependent on adaptive immunity. This suggests that it is a combination of cellular and humoral immunity that mediate the protective effects. Further studies are necessary to extend our knowledge about protective immune responses of atherosclerosis. For instance, nothing is known about the role of other components of adaptive immune responses, such as CD8<sup>+</sup> T cells and NKT, in vaccination-induced atheroprotection.

#### 4.4 SERPINA3 IN ATHEROSCLEROSIS AND ANEURYSM DISEASE (PAPER IV)

Remodeling of extracellular matrix by proteases plays an important role in atherosclerosis [50]. Serine protease inhibitor 3A (serpinA3), also referred to as  $\alpha$ 1-antichymotrypsin [256], is an inhibitor of several proteases involved in this process, such as elastase, cathepsin G and chymase derived from mast cells and neutrophils [230].

We detected an increase in expression of serpinA3n, the mouse orthologue of human serpinA3, in atherosclerotic lesions from immunodeficient *Scid/Scid/Apoe*<sup>-/-</sup> mice compared to lesions from *Apoe*<sup>-/-</sup> mice. *Scid/Scid/Apoe*<sup>-/-</sup> mice display smaller and less inflamed atherosclerotic plaques [131]. In addition, expression analysis by RT-PCR showed 14-fold induction of serpinA3n mRNA levels in atherosclerotic lesions compared to healthy vessels in *Apoe*<sup>-/-</sup> mice. SerpinA3 expression was also detected in human carotid lesions, and was significantly increased in lesions from patients with minor stroke or transitory ischemic attacks (TIA), compared to asymptomatic patients.

SerpinA3 mRNA and protein was found in two of the main cell types of atherosclerotic lesions, namely smooth muscle cells (SMCs) and endothelial cells (ECs). EC-derived expression of serpinA3 was increased by stimulation with TNF $\alpha$ , a pro-inflammatory cytokine shown to accelerate atherosclerosis development [257].

To study the role of serpinA3 in atherogenesis, we generated two transgenic mice overexpressing serpinA3n on a C57Bl/6 background, and crossed one of these with *Apoe*<sup>-/-</sup> mice. SerpinA3n expression in these mice is driven by a human cytomegalovirus immediate early enhancer (HCMVIEE) coupled to the chicken beta-actin promoter. Transgenic mice had 10 times higher gene expression of serpinA3n in aorta, and higher SerpinA3n protein levels in aorta and plasma compared to wild-type (Wt) mice.

We hypothesized that overexpression of serpinA3n would result in attenuated atherosclerotic development, since several of the enzymes it targets are involved in atherogenesis [107, 258, 259]. There was however no difference in atherosclerotic lesion size, neither in aortic root nor in thoracic aorta, between *Apoe*<sup>-/-</sup> mice and *Apoe*<sup>-/-</sup> littermates overexpressing serpinA3n that had been fed a chow diet for 20 weeks. 20 week old *Apoe*<sup>-/-</sup> mice display advanced fibrous plaques, and it cannot be ruled out that

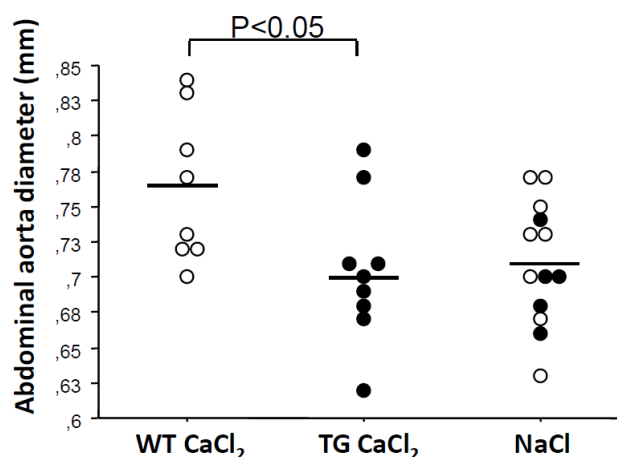


serpinA3n affects early lesion development. It would also be interesting to study if *serpina3n* affects lesion composition; speculatively *serpina3n* overexpression could influence the formation of a more stable plaque phenotype.

Degradation and remodeling of ECM by mast cell- and neutrophil-derived proteases have also been implicated in aneurysm disease [260-266]. Aortic aneurysms are permanent and localized aortic dilatations, and their expansion and rupture results in high morbidity and mortality rates [261]. Like stenotic atherosclerotic lesions, abdominal aortic aneurysm (AAA) accumulate inflammatory cells, and atherosclerosis is a risk factor for AAA [267].

Human AAA samples had a marked decrease in levels of both *serpinA3* mRNA and protein, as compared to control samples from healthy aortas. The difference in *serpinA3n* expression between atherosclerotic lesions and aneurysm samples is in line with the disease-specific aspects of SMC fate. While the AAA lesion is characterized by decreased numbers of vascular SMCs – SMC apoptosis being one of the hallmarks of AAA - there is clear evidence of proliferation of intimal SMCs in the atherosclerotic process. Human AAA samples also contain fewer ECs [268, 269]. Since *serpinA3* also is secreted by the liver as an acute phase plasma protease inhibitor, we examined if vascular disease could alter plasma levels. However, no difference in plasma concentrations was detected between healthy individuals and patients with either AAA or myocardial infarction.

A frequently used method to experimentally induce AAA in a controlled fashion is  $\text{CaCl}_2$ -induced aneurysm in mice [270].  $\text{CaCl}_2$  administration on the abdominal aorta resulted in aorta dilation in Wt C57Bl/6 mice, accompanied by a high elastase and cathepsin G activity, lower amounts of elastin and increased protein expression of mast cell tryptase in the aorta. Mast cell chymase activity was also induced in plasma.



**Figure 8.** Abdominal aorta diameter in wild-type and *serpinA3n* transgenic mice treated with  $\text{CaCl}_2$  or NaCl measured with ultrasound.

Overexpression of serpinA3n suppressed CaCl<sub>2</sub>-induced protease activity, which reduced degradation of elastin. One of the dominant histological features of AAA includes extensive elastin fragmentation [261]. Most importantly, overexpression of serpinA3n completely abrogated aortic dilatation (*Figure 8*). Interestingly, a recent finding showed that administration of recombinant serpinA3n reduced aortic rupture in Angiotensin II-induced AAA formation in mouse [271].

These results support the importance of proteases derived from neutrophils and mast cells in AAA, and illustrate that their inhibition by serpinA3 ameliorates aneurysm disease.

## 5 CONCLUDING REMARKS

It is now generally accepted that atherosclerosis is a chronic inflammatory disease of the arterial wall, and involves both innate and adaptive immunity. It has become clear that the immunology of atherosclerosis includes not only pro-inflammatory processes but also anti-inflammatory and immune-regulatory components. Dissection of immune mechanisms involved in the pathogenesis of this disease is important for development of new immunomodulatory therapies. Mouse models are efficient means of unraveling those mechanisms and have hence been used in the studies of this thesis.

In **paper I** we showed that immunogenic DCs presenting modified LDL induce antigen-specific immunity that augments local inflammation and atherosclerosis development when injected into *Apoe*<sup>-/-</sup> mice. We conclude that DC uptake and presentation of LDL-derived peptides may take part in the progression of the atherosclerotic process.

In **paper II** we demonstrated that ApoB100 presentation by DCs conditioned into a tolerogenic state dampens the inflammatory cellular immune response to ApoB100 and attenuates atherosclerosis when injected into huB100<sup>tg</sup>*xLdlr*<sup>-/-</sup> mice. We conclude that the status of DCs presenting LDL-derived peptides is important for atherosclerosis lesion development.

In **paper III** we utilized different immunodeficient mouse models to demonstrate that the atheroprotection achieved by immunization with modified LDL is dependent on adaptive immunity but not on B cells and antibodies. We conclude that other immune mechanisms besides production of antibodies accounts for the protective effects achieved by vaccination.

In **paper IV** we investigate the role of immune-derived proteases and show that their inhibition by serpinA3 is protective against aneurysm formation, but does not affect atherosclerotic lesion development in *Apoe*<sup>-/-</sup> mice. We conclude that protease inhibition by serpinA3 leads to amelioration of aneurysm disease.

Since it is now evident that atherosclerosis involves autoimmune reactions against LDL particles accumulating in the artery wall, new clinical applications targeting this aberrant antigen specific response would be appealing. The studies presented in this thesis demonstrate that DCs are key players in the immune processes involved in atherosclerosis. In addition, the status of an LDL-presenting DC can be altered to shift the balance from pro-atherogenic immune reactions to anti-atherogenic immune responses. The use of LDL loaded DCs constitutes a potential new treatment strategy for atherosclerosis. Another attractive approach for atherosclerosis treatment is vaccination with LDL. Even though a LDL-derived peptide already has been developed as a vaccine candidate the mechanisms of atheroprotection achieved by immunization is not fully understood. The finding presented in this thesis illustrates that antibodies are not necessary for the protective effects caused by vaccination and further studies could lead to the design of a more effective vaccine. To translate experimental therapies into the clinic it is of great importance to understand the specific inflammatory pathways involved in the disease, and I hope the studies included in this thesis contributed to deeper knowledge about these processes.



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## 7 REFERENCES

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