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LUND UNIVERSITY
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"Once we accept our limits, we go beyond them"

Albert Einstein

"For every minute of anger, you lose 60 seconds of happiness"

unknown

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Summary

The immune system is a promising target for novel therapies that are aiming at reducing cardiovascular diseases. Autoimmune responses against modified low density lipoprotein (LDL) are believed to promote development of atherosclerosis. Proinflammatory immune responses can be counterbalanced by immunosuppressive regulatory T cells (Tregs). Immunizations of hypercholesterolemic mice with oxidized LDL or peptides derived from the protein part of LDL, apoB-100, inhibit the development of atherosclerosis. The protective immunity induced by the apoB-100 peptide vaccine aBp210 is associated with an activation of Tregs indicating that specific activation of Tregs could be a promising target in immune modulating therapies. Activation of the inhibitory Fcγ receptor IIB (FcγRIIB) is an additional potential target for immune modulating therapy as hypercholesterolemic mice deficient in FcγRIIB have a more aggressive disease development. Antigen presentation is a fundamental step in T cell activation. By investigating the role of antigen presentation in atherosclerosis development new targets for intervention could be provided. Antigen presentation on major histocompatibility complex (MHC) class II is critical in activation of CD4+ T cells whereas CD1d antigen presentation is required for activation of NKT cells. The role of MHC class II antigen presentation in atherosclerosis is complex and was unexpectedly found to be associated with reduced atherosclerosis development, whereas, neointima formation in response to vascular injury was accelerated by CD1d lipid antigen presentation. NKT cells and CD1d could therefore be an additional potential target in immune modulating therapies. Except for novel therapies, new biomarkers to better predict development of acute cardiovascular events are also needed in order to reduce the disease. Low levels of circulating Tregs may be a future predictor for myocardial infarction and stroke.

Original Papers

This thesis is based on the following papers, which are referred to in the text by their roman numerals:

I. **Wigren M**, Bengtsson D, Dunér P, Olofsson K, Björkbacka H, Bengtsson E, Fredrikson GN, and Nilsson J. Atheroprotective effects of Alum are associated with capture of oxidized LDL antigens and activation of regulatory T cells. *Circulation Research,* 104(12):e62-70, 2009.

II. **Wigren M**, Kolbus D, Dunér P, Ljungcrantz I, Söderberg I, Björkbacka H, Fredrikson GN, Nilsson J. Evidence for a role of regulatory T cells in mediating the athero-protective effect of apolipoprotein B peptide vaccine. *Journal of Internal Medicine*, in press.

III. Zhao M, **Wigren M**, Dunér P, Kolbus D, Olofsson K, Björkbacka H, Nilsson J, Fredrikson GN. FcγRIIB inhibits the development of atherosclerosis in LDL receptor deficient mice. *Journal of Immunology*, 184, 2253-60, 2010.

IV. **Wigren M**, Söderberg I, Alm R, Ljungcrantz I, Björkbacka H, Fredrikson GN, Nilsson J. Lack of ability to present antigens on MHC class II molecules aggravates atherosclerosis in $ApoE^{-/-}$ mice. *Manuscript*.

V. Ström Å, **Wigren M**, Hultgårdh-Nilsson A, Saxena A, Gomez MF, Cardell S, Fredrikson GN, Nilsson J. Involvement of the CD1d-NKT cell pathway in neointima formation after vascular injury. *Circulation Research*, 101, e83-9, 2007.

VI. **Wigren M**, Björkbacka H, Andersson L, Ljungcrantz I, Fredrikson GN, Persson M, Bryngelsson C, Hedblad B, Nilsson J. Associations of regulatory T cells with cardiovascular disease severity and risk in man. *Manuscript.*

List of papers not included in thesis

Kolbus D, Ramos OH, Berg KE, Persson J, **Wigren M**, Björkbacka H, Fredrikson GN and Nilsson J. CD8+T cell activation predominate early immune responses to hypercholesterolemia in $ApoE^{-/-}$ mice. *BMC Immunology, 2010 Dec 2;11:58*

Kolbus D, **Wigren M**, Ljungcrantz I, Söderberg I, Björkbacka H, Nilsson J and Fredrikson GN. Immunization with cationized BSA inhibits progression of disease in Apobec-1/LDL receptor deficient mice with manifest atherosclerosis. *Immunobiology, 2010 Nov 19 [Epub ahead of print].*

Wigren M and Björkbacka H. Atherosclerosis: Cell biology and lipoprotein. *Curr Opin Lipidol.* 2010 Feb;21(1):97-8.

Nilsson J, **Wigren M**, Shah PK. Regulatory T cells and the control of modified lipoprotein autoimmunity-driven atherosclerosis. *Trends Cardiovasc Med*. 2009 Nov;19(8):272-6.

Kolbus D, Olofsson KE, **Wigren M**, Nilsson J, Björkbacka H and Fredrikson GN. High fat diet induces reduction in regulatory T cells and increased splenocyte proliferation. *Manuscript.*

Kolbus D, **Wigren M**, Ljungcrantz I, Söderberg I, Nilsson J and Fredrikson GN. ApoB-100 peptide immunization do not inhibit advanced lesion progression in Apobec-1/LDL receptor deficient mice. *Manuscript.*

Abbreviations

ACE	Angiotensin converting enzyme
α-GalCer	α-galactosylceramide
APC	Antigen presenting cell
apoB-100	Apolipoprotein B-100
ApoE	Apolipoprotein E
BCR	B cell receptor
BMT	Bone marrow transplantation
cBSA	cationized Bovine Serum Albumine
CD	Cluster of differentiation
ConA	Concanavalin A
cpm	Counts per minute
CTB	Cholera Toxin B subunit
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
CVD	Cardiovascular disease
DAMP	Damage-Associated Molecular Pattern
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorecence Activated Cell Sorting
Fc	Fragment crystallizable
FcγRIIB	Fragment crystallizable gamma receptor II B
FcR	Fragment crystallizable receptor
FCS	Forward Scatter
FMO	Fluorescence minus one
Foxp3	Forkhead box 3
FS	Forward Scatter
GATA	Trans-acting T-cell-specific transcription factor
HDL	High density lipoprotein
HFD	High fat diet

HLA	Human leukocyte antigen
HMG-CoA	3-hydroxy-3-methyl-glutaryl-CoA
HSP	Heat shock protein
ICOS	Inducible T cell costimulator
IDO	Indoleamine-pyrrole 2,3-dioxygenase
IFN	Interferon
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
IMT	Intima media thickness
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
iTreg	inducable regulatory T cell
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
LPS	Lipopolysaccharide
MCP-1	Monocyte chemotactic protein-1
M-CSF	Macrophage colony-stimulating factor
MDCS	Malmö Diet and Cancer Study
MHC	Major Histocompatibility Complex
mmLDL	minimal modified LDL
MOMA	Monocyte-Macrophage
mRNA	messenger Ribonucleic acid
MyD88	Myeliod differentiation factor 88
NF-κB	Nuclear factor κ B
NK cell	Natural Killer cell
NKT cell	Natural Killer T cell
nTreg	natural regulatory T cell
ORO	Oil red O
oxLDL	oxidized low density lipoprotein
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate Buffered Saline
PPR	Pattern recognition receptor
ROS	Reactive oxygen species
SCID	Severe immunodeficiency
ScR	Scavenger receptor

SMC	Smooth muscle cell
SS	Side Scatter
SSC	Side Scatter
STAT	Signal Transducer and Activator of Transcription
Tbet	T-cell-specific T-box transcription factor
TCR	T cell receptor
Tfh	T follicular helper
TGF	Transforming growth factor
Th	T helper
TIA	Transient ischemic attack
TLR	Toll-like receptor
TNF	Tumor Necrosis Factor
Tr1	Type 1 regulatory T cell
Treg	Regulatory T cell
V(D)J	Variable (Diverse) Joining
VCAM-1	Vascular Cell Adhesion Molecule-1
WT	Wild type

Introduction

More than fifteen years ago, researchers tried to induce atherosclerosis in rabbits by immunizing with low density lipoprotein (LDL)[1, 2]. However, in contrast to what was expected, the rabbits showed reduced development of atherosclerotic lesions. This finding suggested that it could be possible to induce an atheroprotective immune response with a vaccine. The search for the appropriate antigen started and the research at the Experimental Cardiovascular Research Unit at Lund University has focused the attention on peptide fragments from apoB-100, the major protein in LDL. A number of apoB-100 related peptides were identified as atheroprotective when used together with Alum as a vaccine in hypercholesterolemic mice[3-5]. The aim of this research is to develop new therapies in order to fight cardiovascular diseases that are the most common cause of death in the world. The first clinical trial with vaccinations against atherosclerosis with an apoB-100 derived peptide will most likely start during 2011.

Aims of the thesis

Earlier studies from our group have shown that immunizing hypercholesterolemic mice with apoB-100 derived peptides and Alum as adjuvant are associated with a reduction in atherosclerosis development. In addition, it has been shown that Alum, without any antigen, also induces atheroprotection. The immune-modulating mechanism involved in this atheroprotection is not yet fully elucidated. Before starting clinical trials with the apoB-100 vaccine it will be important to understand the mechanism of action to create a vaccine that is as efficient as possible. The specific aims of the studies in Paper I and II were:

- To investigate the mechanism involved in the atheroprotective effect of Alum.
- To study the possibly role of regulatory T cells in atheroprotection induced by the prototype vaccine aBp210.

Furthermore, the immunological mechanisms involved in the atherosclerotic process are not fully elucidated. Discovering how specific immune mechanisms are involved in the development of atherosclerosis could provide new targets for intervention. The specific aims of the studies in Paper III, IV and V were:

- To investigate the role of the inhibitory Fc receptor FcγRIIB in atherosclerosis development.
- To study how the antigen presenting molecules MHC class II and CD1d are involved in atherosclerosis development and neointima formation, respectively.

Besides developing new therapies to fight atherosclerosis, it is also of great importance to improve the tools to use in predicting the risk for development of a myocardial infarction and stroke. Therefore, the aim of the study in paper VI was:

- To determine if circulating regulatory T cells can predict cardiovascular events in humans.

Background

The immune system

The immune system protects us from infectious agents such as bacteria and viruses as well as potentially harmful modified self-antigens that could cause autoimmunity. The immune system can be divided into two different branches; the innate and the adaptive immune system. The innate immune system is represented by mechanical and chemical barriers, activation of the complement system and by the innate immune cells. Dendritic cells (DCs), granulocytes, natural killer (NK) cells, mast cells, monocytes and macrophages are innate immune cells and they are programmed to detect foreign or modified molecules of exogenous or endogenous origin. The adaptive immune system, which evolved later than the innate immune system, is composed of specialized antigen-specific lymphocytes called T and B cells. T and B cells require presentation of antigens and signals from innate immune cells to become activated. Once the adaptive immune cells have been activated, there will be memory cells generated to rapidly respond in case of a new infection by the same pathogen. Although T and B cells express receptors with enormous diversity, they still depend on antigen presentation, cytokine and chemokine secretion as well as expression of costimulatory molecules from innate immune cell to become fully activated[6, 7]. This fact emphasizes the importance of both innate and adaptive immunity in generating an effective immune response.

Innate immunity

The innate immune cells are programmed to detect foreign molecules like the endotoxin lipopolysaccharide (LPS) or viral DNA. Modified self-antigens, such as oxLDL, are also detected by the innate immune system[8]. These foreign or modified molecules are collectively called pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) and they are detected by pattern recognition receptors (PRRs) like Toll like receptors (TLRs) and Scavenger receptors (ScRs)[7, 9]. PRRs are mainly expressed on the innate immune cells. Monocytes, macrophages and dendritic cells are professional antigen presenting cells (APCs) and they internalize antigen via either phagocytosis or endocytosis[10]. Monocytes are large circulating cells with the potential to produce huge amounts of cytokines and to phagocytose antigens. Upon tissue infiltration monocytes will differentiate into

macrophages or DCs. Monocytes can phagocytose antigens either directly or via opsonisation (when the antigen is covered by antibodies or complement to make phagocytosis more effective). Monocytes can be divided into inflammatory and anti-inflammatory subsets with different expression of surface molecules and with different response to a certain stimuli[11]. In humans, the inflammatory monocytes are described as CD16- and the anti-inflammatory monocytes as CD16+[12]. Further subdivisions of the human monocytes can be done but this will not be further discussed here. In mice, the monocyte populations can be classified upon their expression of Ly-6C. Ly-$6C^{high}$ monocytes are called inflammatory and Ly-$6c^{low}$ are termed resident monocytes. Ly-$6c^{high}$ monocytes dominate in early inflammation and will give rise to M1-type or classically activated macrophages. Ly-$6c^{low}$ monocytes patrol the vasculature and will differentiate into M2-type or alternatively activated macrophages in the tissue[13]. Macrophages reside within tissues and when activated they will remove cell debris and antigens via phagocytosis. Furthermore, activated macrophages produce large amounts of proinflammatory cytokines as TNF-α, IL-1, IL-12 and IL-18 directing the naive T cells into the Th1 subtype[6, 14]. Monocytes and macrophages will be further discussed later in the context of atherosclerosis. DCs reside in the tissues as immature DCs where they constantly scan for antigens. The scanning is performed by the PPRs and when a DC encounters an antigen and PPRs simultaneously are stimulated on the DC it will become activated and start to migrate to a lymph node were it can present antigens to T cells[15]. DCs present exogenous peptide antigens on major histocompatibility complex (MHC) class II molecules, intracellular antigens are presented on MHC class I and lipid antigens are presented on CD1d[16]. When DCs become activated they up regulate their expression of the costimulatory molecules CD80, CD86 and CD40 that are important in activation, as well as inactivation, of T cells. Other costimulatory or coinhibitory molecules on APCs that are involved in activation of T cells are ICOS ligand and OX40 ligand[17]. Activated DCs have the ability to produce several different chemokines and cytokines, IL-6, IL-12 and TNF-α being some of them[6]. The specific antigen encountered by the DC will determine the nature of the immune response the DC will initiate. It is the combinations of the cytokines produced and the costimulatory molecules expressed that is directing the different types of T and B cell responses[18]. T and B cell immune responses will be further discussed below.

TLRs and scavenger receptors

TLRs and ScRs are examples of PRRs. There are at least 10 and 13 known different human and mouse TLRs respectively, and they are mainly expressed on innate immune cells but TLRs can also be found on non-immune cells such as smooth muscle cells and endothelial cells[19]. TLR ligands are mainly conserved microbial structures e.g. LPS and microbial DNA, but may also have endogenous origin[20]. TLRs

function as dimers, mostly homodimers but in some cases also as heterodimers[21]. Ligand-binding to TLRs leads to an inflammatory response with NF-κB activation and IL-1β secretion as well as to increased expression of costimulatory signals on APCs[22]. The intracellular signal transduction pathways in TLR signaling involves four different adaptor proteins, myeloid differentiation factor 88 (MyD88) being one of them[20, 23]. Different combinations of these adaptors results in activation of specific sets of transcription factors regulating several pro-inflammatory genes. Scavenger receptors are important in removing foreign or altered self-molecules such as oxLDL and apoptotic bodies. Activation of scavenger receptors does usually not induce NF-κB activation and secretion of cytokines/chemokines[24]. CD36, ScR-A and ScR-B1 are examples of ScRs important in atherosclerosis and will be further discussed later.

Fc receptors

The Fc region of antibodies (immunoglobulins) binds to Fc receptors (FcRs) present on innate immune cells and B cells. T cells do not express FcRs[25]. Several effector functions of the innate immune cells depend on FcR activation. The FcRs specific for IgG, IgE and IgA are named FcγR, FcεR and FcαR, respectively. FcγR signaling can be either activating or inhibitory depending on the intracellular signaling domain involved. Signaling via ITAMs leads to activation of the cell as opposed to ITIM activation that leads to inhibitory signals. Binding to activating FcRs promotes phagocytosis of opsonised antigens leading to clearance of the antigen but also activation of the phagocyte. Activated phagocytes secrete anti-microbial molecules resulting in destruction of pathogens in the surrounding area. The inhibitory FcγRIIB is the only FcR expressed on B cells. FcγRIIB signals via ITIMs leading to feedback inhibition of B cell activation and antibody production[6, 26, 27].

Adaptive immunity

T and B cells differ from the innate immune cells in the diversity of their antigen receptors. To be able to protect the host from all potential antigens, both pathogenic and self-derived, the adaptive immune system has to be extremely diverse. V(D)J recombination (also called somatic rearrangement) is the key to the nearly infinite number of different T cell receptors (TCRs) and B cell receptors (BCRs). During development, each lymphocyte undergoes V(D)J recombination resulting in a structurally unique receptor[28]. Both T and B cells are produced in the bone marrow. T cells then mature in the thymus and B cells continue their maturation in the bone marrow. A mature T or B cell that has not yet encountered its' antigen is called a naïve lymphocyte. The naïve lymphocytes are mainly present in lymph nodes, spleen and mucosal tissues waiting to meet their respective antigen. T and B cells recognize antigens in two different ways. T cells recognize peptide fragment of antigens

presented on MHC class I or II molecules on APCs. B cells on the other hand recognize free antigens of any biochemical origin; protein, lipids, polysaccharides and nucleic acids. When naïve T and B cells encounter their antigen they will differentiate into effector cells[6].

T cells subsets

T cells are divided into several different subsets based on the expression of surface markers and the TCR formation; αβ T cells, γδ T cells and NKT cells. All T cells express the CD3 molecule that is the signaling part of the TCR[29]. γδ T cells are a small subset of T cells expressing the γ- and δ-chains in their TCR, instead of the far more common α- and β-chains. γδ T cells are common in mucosal barriers such as the gut and the lungs[30]. γδ TCRs are termed non-conventional or innate-like T cells because of their recognition of conserved non-peptide antigens produced by stressed and damaged cells[31]. γδ T cells will not be further discussed. NKT cells are yet another subset of innate-like T cells that express characteristics of both T cells and NK cells. NKT cells have TCRs but unlike conventional T cell their TCR reacts with lipid or glycolipid antigens presented on the MHC class I-related CD1 molecule. Mice only express CD1d, humans on the other hand have four different CD1 molecules, CD1a-d. Activation of NKT cells leads to a rapid response including production of both pro- and anti-inflammatory cytokines[32,33]. Conventional T cells, or αβ T cells (from now on only named T cells), represents the largest subset of T cells in the immune system. T cells are divided into CD4+ and CD8+ depending on which of the two co-receptors they express. CD4+ T cells recognize peptide-antigens presented on MHC class II molecules. CD8+ T cells, on the other hand, need antigen presentation on MHC class I to become activated. MHC-peptide interaction with the TCR is often referred as signal 1 in activation of T cells but it is not sufficient to obtain a fully activated T cell. Costimulatory or coinhibitory signals between the T cell and the APC are required for complete activation of the T cell and this is usually named signal 2[6, 34]. Costimulation and coinhibition will be further discussed below.

CD8+ T cells

Cytotoxic CD8+ T cells participate in the immune responses against intracellular bacteria, viruses, parasites and cancer cells. They are activated by peptide-antigens presented on MHC class I molecules. MHC class I is expressed by all nucleated cells and proteins are continuously being degraded in the cells by the proteosomes and the peptides generated are loaded on MHC class I. Peptides presented on MHC class I mirror the protein metabolism inside the cell leading to presentation of virus-peptides of virus infected cells and tumor-peptides of tumor cells et cetera. Foreign peptides presented on MCH class I will activate cytotoxic CD8+ T cells. Activation of CD8+

T cells also requires costimulation via CD28 (on the T cell) and CD 80 or CD86 (on the APC) and secretion of cytokines from CD4+ T cells[35]. Following activation, CD8+ T cells will start to express Fas ligand (FasL) and to release the cytotoxins perforin and granzymes. Interaction between FasL and Fas, expressed on the target cells, will induce a caspase cascade in the target cell leading to apoptosis. Perforin is a cytolytic protein that by polymerizing and insertion of itself in the target cell it forms a pore that allows water and ions to enter the cell leading to osmotic swelling and cell death. Granzymes are proteases that can enter the target cell via the pore formed by perforin. Inside the target cell, granzymes will activate the caspase cascade leading to apoptosis. Moreover, activated CD8+ T cells produce large amounts of IFN-γ, leading to activation of macrophages[36].

CD4+ T cells

CD4+ T cells are also called T helper (Th) cells and these cells are key players in orchestrating immune responses. The Th cells have traditionally been subdivided into two different effector populations; Th1 and Th2 cells. Th1 cells activate macrophages and CD8+ T cells and secrete proinflammatory cytokines such as IFN-γ and TNF-α. Th2 cells, on the other hand, produce IL-4, IL-5, IL-6, IL-10 and IL-13. These cytokines stimulates B-cells to proliferate and produce antibodies, leading to activation of the humoral immune response. This traditional subdivision of Th cells is nowadays too simple to reflect the complex nature of Th cells. Several new CD4+ T effector cells, as well as CD4+ T cells with immune regulatory capacity, has been discovered since the Th1/Th2 dogma was presented in the 1980-ies[37, 38]. Meanwhile new CD4+ T cell populations has been identified, plasticity between different subgroups of CD4+ cells has also been discovered[39]. The fate of a naïve Th cell is believed to be determined by the cytokines and costimulatory or coinhibitory signals that are present upon activation. T cells are activated by APCs, primarily DCs, that release a mixture of cytokines and express costimulatory or coinhibitory molecules that will activate the T cell[18, 34]. The nature of the antigen determines the cytokine profile and the pattern costimulatory and coinhibitory signals expressed by the APC and consequently also the T cell response that is initiated. The CD4+ T cell population is divided into Th1, Th2, Th3, Th9, Th17, Tr1, natural regulatory T cells (Tregs) and T follicular helper cells (Tfh). Each cell type expresses a specific combination of extra- and intracellular markers and/or produces certain cytokines. Th1, Th2, Th9, Th17 and Tfh cells are considered to promote inflammation while Th3, Tr1 and nTregs are immune regulatory cells with the potential to reduce and inhibit immune responses. However, the lineage commitment seems not always to be permanent and studies have shown that regulatory T cells can become disease-inducing or pathogenic autoreactive Th1 cells under certain conditions[40]. Th1, Th2

Th17, Tregs and NKT cells will be further described below and are overviewed in Figure 1.

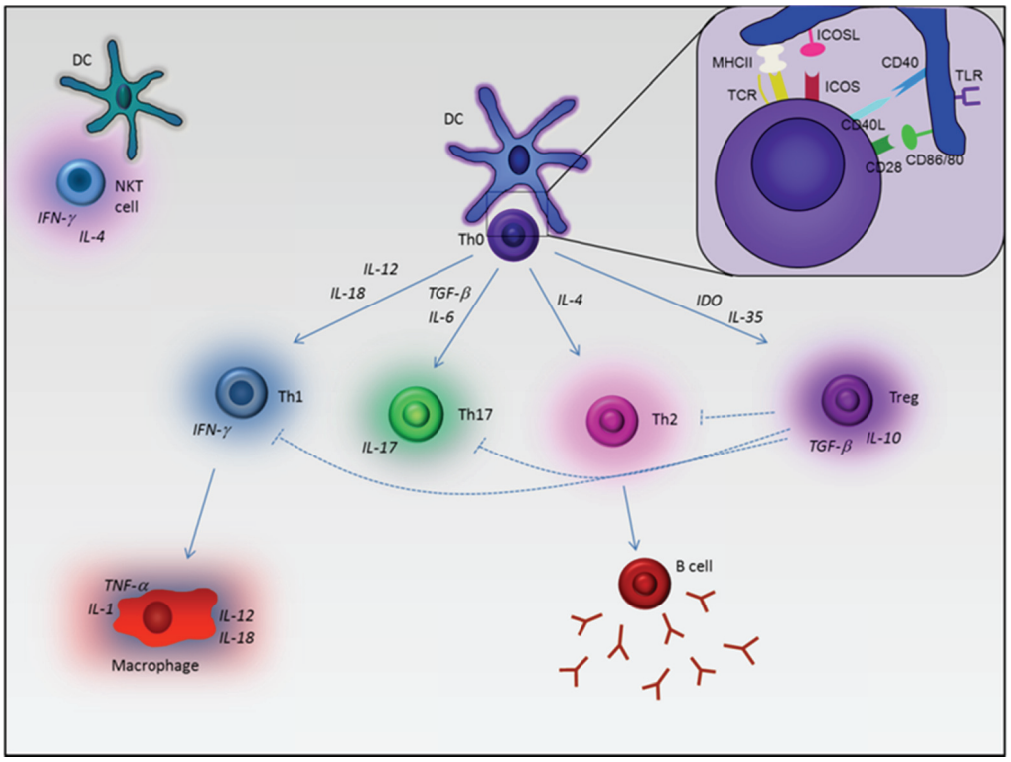


Figure 1. T cell subclasses. Antigens are presented to Th cells by MHC class II (MHCII) molecules on Dendritic cells (DCs). T cells with a TCR recognizing the antigen will become activated. Beside the TCR-MHC class II interaction, activation of a naive T cell requires costimulation. The costimulatory molecules CD80 and CD86 on APCs interact with CD28 on T cells. CD80/86–CD28 interaction is required for any T cell activation. CTLA-4 is a coinhibitory molecule that interacts with CD80 and CD86. In contrast to CD28, CTLA-4 provides an inhibitory signal leading to an inactivation of T cells. DCs cells also express ICOS ligand (ICOSL), which is a costimulatory molecule of the same family as CD80 and CD86. ICOS expressed on T cells interacts with ICOSL. DCs cells also express CD40 that interacts with CD40L on T cells. The antigen taken up by the DC determines the pattern of costimulatory molecules and cytokines secreted from the APC. This pattern will determine the further fate of the naive T cell. Differentiation into Th1 cells results in an inflammatory immune response. IFN-γ is an important cytokine in Th1 cell differentiation and function leading to activation of macrophages. CD40–CD40L interaction, ICOSL–ICOS interaction, and IL-4 secretion are characteristic of a Th2 cell response. Differentiation into Th2 cells leads to activation of B cells. B cells will secrete antigen-specific IgG that help to clear the antigen. Antigens presented on DCs can also induce T cells with a regulatory phenotype. Regulatory T cells are immune inhibitory cells that dampen immune responses as they secrete the anti-inflammatory cytokines IL-10 and TGF-β. Th17 cells have similarities with both Th1

and Th2 cells but are regarded as a distinct population of T helper cells and their differentiation is mainly driven by TGF-β and IL-6. Activated Th17 cells produce the proinflammatory cytokine IL-17. The innate-like NKT cells are activated by lipid antigens presented on CD1 by DCs. Activated NKT cells can produce both pro- and anti-inflammatory cytokines.

Th1 cells

Secretion of IL-12 and IL-18 from APCs promotes naïve CD4+ T cells to express the Th1 specific transcription factors T-box expressed in T cells (Tbet) and signal transducer and activator of transcription 4 (STAT4) leading to differentiation into Th1 cells. Activated Th1 cells produce the proinflammatory cytokines IFN-γ, TNF-α and TNF-β leading to activation of macrophages. IL-2 secreted by the Th1 cells and interactions between the costimulatory receptor CD40, present on APCs, and the ligand CD40L on T cells, further promotes activation and proliferation of Th1 cell as well as CD8+ T cells. IFN-γ produced by the Th1 cells inhibits the differentiation of naïve T cells into Th2 cell[39, 41]. Activated macrophages have an enormous capacity of engulfing opsonized pathogens leading to clearance of an infection. A Th1 response is important in protecting the host from intracellular pathogens but prolonged activation of Th1 cells leads to tissue damage and inflammatory diseases.

Th2 cells

Th2 cells produce several cytokines, including IL-4, IL-5, IL-13 and IL-25. IL-4 has an important role in initiating Th2 differentiation through its action on STAT6. This signal molecule induces up-regulation of the transcription factor Trans-acting T-cell-specific transcription factor-3 (GATA3), which is the master regulator of Th2 cell differentiation. Th2 cytokines inhibits the action of IFN-γ including the activation of Th1 cells and macrophages. As a result of this, Th2 cell responses are not associated with a large activation of macrophages as Th1 cell immune responses are[42, 43]. Th2 cells are, on the other hand, associated with B cell activation and differentiation into plasma cells, leading to increased antibody production and class switch. The combined Th2 and B cell response is important in fighting extracellular pathogens. IL-33 is a more recently discovered cytokine associated with a Th2 response that has the potential to increase production of other Th2 cytokines and to promote antibody production[44]. Th2 cells have been implicated in several allergic diseases such as asthma and rhinitis[39].

Th17 cells

IL-17 secreting Th17 cells are a relatively recently discovered T helper cell subset that is believed to be mainly proinflammatory. Th17 cells seem to have similarities with

both Th1 and Th2 cells but are regarded as a distinct population of T helper cells[39]. Differentiation into Th17 cells is mainly driven by TGF-β and IL-6, but IL-21, and IL-1β also appear to influence Th17 differentiation. IL-23 is required for the development of pathogenic Th17 cells but since naïve T cells do not express the IL-23 receptor an initial activation with TGF-β and IL-6 is required. Activated Th17 cells produce the proinflammatory cytokines IL-17 and IL-21. IL-17 induces tissue inflammation and is therefore commonly associated with autoimmune and allergic responses. Both IFN-γ and IL-4 inhibits Th17 differentiation[45, 46].

Regulatory T cells

Regulatory T cells (Tregs) have immunosuppressive activity and their major function is to maintain self-tolerance and immune homeostasis. Tregs recognize self-derived antigens and in contrast to Th1, Th2 and Th17 cells, Tregs secrete large amounts of inhibitory cytokines such as TGF-β and IL-10 when activated. However, Tregs with specificity for foreign antigens have also been reported[47]. Deficiency of Tregs results in severe autoimmune disorders[48]. There are several different subsets of Tregs; natural Tregs (nTregs) and induced Tregs (iTregs, also named adaptive Tregs) which include Th3 and type 1 regulatory T (Tr1) cells as well as peripherally generated Foxp3+ T cells[39]. nTregs are generated in the thymus and are defined based on their expression of CD4, CD25 and Foxp3. The mechanism by which nTregs suppresses effector T cells is partly unknown but both cytokine secretion and cell-cell contact dependent mechanisms seems to be involved in suppressing pathogenic or autoimmune responses. The ability of Tregs to consume large amounts of IL-2 and thereby to inhibit the survival of effector cells represents another possible mechanism of nTreg suppression[49, 50]. IL-35 is a new inhibitory cytokine believed to be produced by nTregs and to contribute to their suppressive activity. nTregs can start to proliferate in response to IL-35 and proinflammatory Th17 cells are suppressed by IL-35[51]. The target cells of nTregs are in principal all other immune cells, including different T effector cell subsets, B cells and APCs. It has been shown that nTregs are recruited to sites of Th1 and Th17 mediated autoimmune responses presumably to limit the inflammation[52]. The Tr1 and Th3 iTregs are developed in the periphery in response to specific signals and cytokines from DCs. Specific subsets of tolerogenic DCs have the ability to produce the immune regulatory enzyme indoleamine 2,3-dioxygenase (IDO) that catabolizes the amino acid tryptophan and generates tryptophan metabolites. T effector cell proliferation is dependent on tryptophan and IDO can thereby induce specific down regulation of inflammatory responses[53]. Furthermore, the tryptophan metabolites produced by IDO promotes differentiation and activation of iTregs[54]. Tr1 and Th3 cells produce large amounts of the immunosuppressive cytokines IL-10 and TGF-β, respectively. Secretion of TGF-β and IL-10 from Tregs cause inactivation of APCs leading to reduced antigen presentation capacity of the

APCs to T effector cells, a mechanism called bystander immune suppression[47]. Tregs are overviewed in Figure 2.

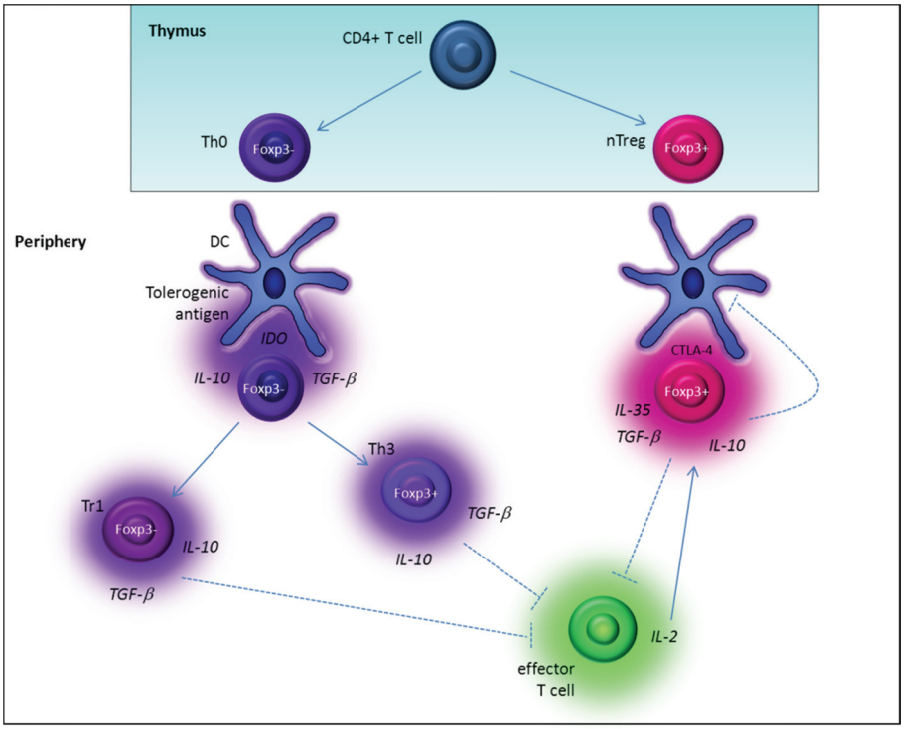


Figure 2. nTregs and iTregs. nTregs are developed in the thymus and expresses Foxp3+. Th0 cells can differentiate into Tr1 or Th3 Tregs if activated by a tolerogenic antigen via DCs. Both nTregs and iTregs produce the anti-inflammatory cytokines Il-10 and TGF-β that inhibits other immune cells such as effector T cells. nTregs can also induce suppression via IL-35 and via consumption of IL-2.

NKT cells

NKT cells are a specific subset of innate-like T cells that express NK cell markers and TCRs. However, the diversity of the TCR on NKT cells is limited compared to conventional T cells. In contrast to conventional T cells that recognize peptide antigens, NKT cells recognize lipid and glycolipid antigens presented on the MHC class I-related CD1 molecule on APCs. Upon stimulation, NKT cells secrete large amounts of cytokines, both pro- and anti-inflammatory ones. IFN-γ, IL-4, IL-5, IL-10, IL-12 are all secreted by activated NKT cells[55]. NKT cells may express CD4 or CD8 but a population of double negative (CD4-CD8-) cells also exists [56]. NKT cells respond rapidly when activated by their antigens but do not develop into memory

cells. NKT cells can mediate both pro- and anti-inflammatory immune responses and they act as a bridge between innate and adaptive immunity [32].

Costimulation and coinhibition

The full activation of a T cell requires costimulatory signals from an APC. In order to reduce or inhibit an immune response, coinhibitory molecules are expressed on the APC. Costimulation and coinhibition is the result of signaling between a specific receptor and its ligand, one present on the T cell and the other on an APC. Costimulation and coinhibition helps to "fine tune" the immune response in order to achieve the proper response to a certain antigen. One of the most studied costimulatory receptor is CD28 and its ligands CD80 and CD86 (also called B7-1 and B7-2 respectively)[57]. CD28 is constitutively expressed on most T cells and signaling via CD28 and CD80 or CD86 on APCs induces IL-2 production and proliferation of T cells. Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) is also signaling via CD80 and CD86 but as opposed to CD28, it will induce coinhibitory signals in the T cell. CTLA-4 is expressed on activated T cells and via interaction with CD80 and CD86 it inhibits T cell activation. CTLA-4 competes with CD28 for binding to CD80 and CD86 but CTLA-4 binds with higher affinity than CD28[6]. Deficiency of CTLA-4 results in systemic autoimmunity reflecting the importance of coinhibition in regulating T cell immune responses[58]. CTLA-4 reduces the immune stimulatory activity of DCs by down regulation of CD80 and CD86 on the APC [58]. Inducible costimulatory (ICOS) is expressed on recently activated effector T cells but not on naïve T cells. ICOS signals via ICOS ligand expressed on APCs and this signaling pathway seems important in both Th2 cells and Treg activation. ICOS-deficiency is associated with development of autoimmune diseases[17]. CD40 ligand (CD40L) is another costimulatory molecule rapidly induced on T cells upon activation. It binds CD40 on APCs leading to up-regulation of CD80 and CD86, further enhancing the T cell activation. Furthermore, CD40-CD40L interaction is important in B cell class switching[17]. OX40 is a yet another costimulatory molecule expressed on activated T cells and OX40-OX40 ligand (OX40L) signaling promotes a long-lasting T cell response. It enhances survival of memory T cells and inhibits development of Tregs[59]. OX40 signaling provides B cell help since OX40L is expressed on B cells leading to antibody production and isotype class switch[60]. Several other costimulatory and coinhibitory molecules exists but will not be further discussed here.

Cardiovascular diseases and atherosclerosis

Cardiovascular disease (CVD) is the number one cause of death in the world and the number is increasing [61]. In Sweden, 41% of all deaths in 2008 were caused by CVD[62]. Coronary heart disease is the single most common cause of death and stroke is the second. The risk factors of CVD are more or less reflecting our way of living. Tobacco smoking, physical inactivity, hypertension and dyslipidemia are the main risk factors for developing a myocardial infarction. Diabetes, age, gender and genetic background also play important roles[62]. The underlying cause of most CVD is atherosclerosis. The disease starts early in life but the symptoms appear much later, often without any previous warning signs. Since CVD still is increasing, the tools available to detect and treat the disease are not sufficient. Cholesterol lowering drugs, primarily the statins, are the main treatment for CVD, but since the treatments available today only prevent 4 out of 10 cardiovascular events there is still a large need for new therapies to prevent CVD.

Atherosclerosis – an inflammatory disease

Atherosclerosis is an inflammatory disease of large and medium-sized arteries. The atherosclerotic process is initiated at an early age with the formation of fatty streaks. Fatty streaks are accumulations of lipid filled macrophages, so called foam cells, as well as a few numbers of T cells in the intimal layer of the artery. Fatty streaks may disappear over time or in the presence of hypercholesterolemia, it can progress into a mature atherosclerotic lesion. During hypercholesterolemia, low density lipoprotein (LDL) particles which are the main cholesterol carriers in blood, can be entrapped in the intimal part of the vessel wall[63]. Entrapped LDL can be modified by enzymes or reactive oxygen species (ROS) leading to the generation of minimal modified LDL (mmLDL) and oxLDL. The modified LDL particles are immunogenic and start an inflammatory process within the vessel wall. The inflammatory process is complex and involves a number of different immune cells as well as several antigens[64]. Modified LDL trapped inside the intima activates endothelial cells via release of phospholipids and other reactive lipid metabolites leading to expression of leukocyte adhesion molecules like vascular-cell adhesion molecule 1 (VCAM-1) and production of monocyte chemoattractant protein-1 (MCP-1). Activation of endothelial cells can be enhanced by other proinflammatory stimuli such as hyperglycemia, hypertension and cigarette smoking[65]. An activated endothelium reduces the speed of leukocytes making them roll and subsequently pass through the endothelium into the subendothelial space. Leukocyte migration into the subendothelial space is also dependent on expression of L- and P-selectin on the leukocytes. Selectins bind to the adhesion molecules resulting in reduced speed and subsequently to migration from

the blood stream into the subendothelial space[24]. Hypercholesterolemic mice deficient in VCAM-1 or selectins have reduced lesion size demonstrating the importance of leukocyte adhesion to the endothelium in atherosclerosis development[66,67]. Atherosclerotic lesions are predominantly formed at sites of altered blood flow such as low shear stress or increased turbulence. The altered blood flow changes the expression of adhesion molecules and inflammatory genes in the vascular wall increasing the probability of lesion formation at these sites[68]. Monocytes and T cells adhere to VCAM-1 leading to migration of these cells into the inflamed intima. Activated endothelial cells and smooth muscle cells produce macrophage colony-stimulating factor (M-CSF) which promotes differentiation of monocytes into macrophages[24]. M-CSF is a proatherogenic cytokine and M-CSF deficiency in mice reduces atherogenesis[69]. The differentiation of monocytes into macrophages is associated with an increased expression of ScRs and TLRs as well as cytokine production and release. LDL is normally taken up by cells via binding to the LDL receptor but oxLDL have lost its ability to bind to LDL receptors. Instead, oxLDL binds to ScRs, such as CD36, on macrophages leading to macrophage cholesterol uptake and accumulation[24]. ScRs are important in clearance of potentially harmful molecules and materials. However, deficiency of ScRs in hypercholesterolemic mice has shown conflicting results on atherosclerosis development indicating a complex role of ScRs in innate immunity and atherosclerosis[20]. A macrophage with accumulated cholesterol in the cytosol is called a foam cell, which is a cell that is characteristic for atherosclerosis. mmLDL bind TLRs which, in contrast to ScR binding, leads to activation of an inflammatory response in the macrophage with NF-κB activation and proinflammatory cytokine and chemokine release[24]. Deficiency of TLRs or their signaling adaptor molecule MyD88 has been shown to reduce atherosclerosis development in mice[70,71]. This emphasizes the critical role of innate immunity in the disease. The cytokines and chemokines released from macrophages participate in activation of T cells and causes additional cytokine and chemokine release. Chronic inflammation of the vascular wall will activate medial smooth muscle cells (SMCs) to migrate into the lesion and start to proliferate to form the fibrous cap. The fibrous cap will cover the mixture of lipids, immune cells and debris that forms the core region of the lesion[64].

T and B cells in atherosclerosis – proatherogenic or antiatherogenic?

The hypothesis of atherosclerosis as an inflammatory disease was actually proposed by Virchow already in 1856[72]. However, the important role of the adaptive immune system in this inflammation did not begin to be revealed until in the late 1980s when it was found that MHC class II frequently is expressed in human lesions[73]. It was later shown that T cells, both CD4+ and CD8+, and small numbers of B cells and NKT

cells also are present in the lesions, particularly in the shoulder regions and close to the fibrous cap[74]. Modified LDL particles, but also other antigens such as heat shock proteins (HSP) are believed to be important antigens in T cell activation in atherosclerosis. Modified LDL is taken up by APCs and LDL related antigens are presented to T cells after being processed and loaded on MHC. Most of the T cells in atherosclerotic plaques are of the proinflammatory Th1 type. However, Tregs may also be activated by the self-derived antigens counterbalancing the Th1 immune response.

The role of Th1 cells in atherosclerosis

CD4+ T cells are considered as proatherogenic. This assumption is based on studies with hypercholesterolemic severe immunodeficient (SCID) mice to which CD4+ T cells were transferred resulting in increased atherosclerosis development[75]. Moreover, hypercholesterolemic mice deficient in CD4+ T cells have reduced lesion size further indicating that CD4+ T cells are proatherogenic[76]. The proinflammatory cytokines IL-12 and IL-18 are produced by monocytes and macrophages in the atherosclerotic lesion thereby attracting the CD4+ cells to the vessel wall[77]. T cell activation in atherosclerosis may take place in the plaque or in regional lymph nodes, possible as a consequence of APC migration from the plaque to a lymph node[24]. T cells activated in lymph nodes will then migrate into the plaque where they again will encounter their specific antigens. It has been shown that about 10% of the T cells extracted from human lesions are specific to oxLDL and secrete IFN-γ in response to activation, indicating that a proinflammatory Th1 immune response to oxLDL is present in the plaque[78]. Activated Th1 cells start to express CD40L and produce IFN-γ and TNF-α resulting in further activation of monocytes and macrophages including increased production of proinflammatory cytokines resulting in enhanced inflammation. The proatherosclerotic role of Th1 cells have been confirmed by studies in experimental animals. When the Th1 transcription factor Tbet or the Th1 cytokine IFN-γ were knocked out in hypercholesterolemic animals, atherosclerosis was reduced[79, 80]. Vaccination against the Th1 cytokine IL-12 results in a blockade of endogenous IL-12 and reduced atherogenesis[81]. Furthermore, treatment with Th1 cytokines leads to increased atherosclerosis and IFN-γ strongly inhibits collagen production and smooth muscle cell proliferation in the vascular wall leading to more unstable lesions and increased risk of plaque rupture[69,24]. Taken together, these results suggest that reduction of Th1 cell activity in the atherosclerotic lesions will result in less inflamed and more stable plaques. Potential proatherogenic and antiatherogenic effects of Th1 cells and Tregs are overviewed in Figure 3.

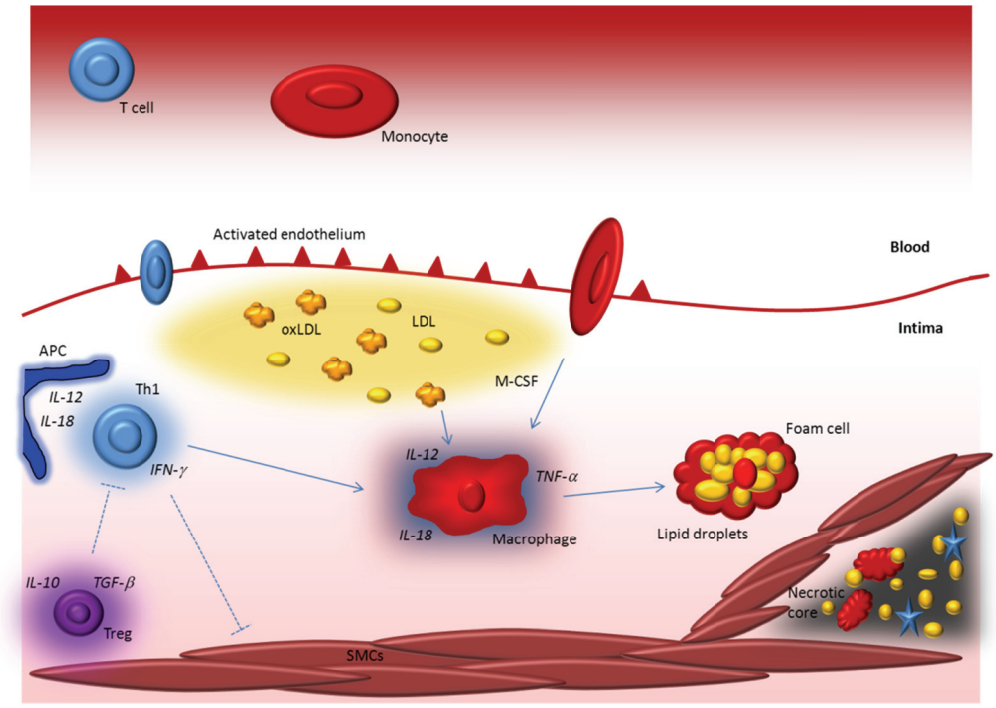


Figure 3. Potential proatherogenic and antiatherogenic effects of Th1 cells and Tregs in atherosclerosis. Th1 cells promote plaque inflammation and destabilization via release of IFN-γ. IFN–γ activates macrophages and prevents infiltration of SMCs. Tregs have the potential to inhibit Th1 cells and therefore prevent further development of the plaque.

Th17 cells - a new player with pleiotropic function?

The more recently discovered Th17 cells, and their signature cytokine IL-17, are involved in several autoimmune diseases. However, the role of Th17 cells in atherosclerosis has not yet been fully elucidated. Results from both human and murine studies show conflicting results making interpretation difficult. T cells from atherosclerotic human coronary arteries produce more IL-17 in response to polyclonal activation compared to cells from non-diseased vessels[82]. Moreover, expression of IL-17 is up-regulated in plaques from symptomatic patients compared to plaques from asymptomatic patients[83]. Hypercholesterolemic mice deficient for the IL-17 receptor[84] or when IL-17 is neutralized by a blocking antibody are protected against atherosclerosis development[85, 86]. Furthermore, treatment with exogenous IL-17 has been shown to aggravate atherosclerosis in mice[86]. Conversely, experimental studies with IL-17 in vivo administration have also been shown to reduce lesion formation and neutralization of IL-17 was in the same report associated with increased

atherosclerosis[87]. In conclusion, the role of Th17 cells and IL-17 in atherosclerosis is controversial and it is likely that Th17 cells have a pleiotropic function. Further studies on the role of Th17 cells and their signature cytokines in atherosclerosis is required to determine if these are potential new targets in therapies aiming at reduce atherosclerosis.

Th2 cells and B cells – protective or not?

CD4+ Th2 cells are also present in the atherosclerotic lesions and they are generally described as atheroprotective[88]. The atheroprotective effect of Th2 cells is believed to be related to the fact that Th2 cytokines are antiatherogenic and counteracts the action of Th1 cytokines[69]. Furthermore, a Th2 cell response is associated with B cell activation and antibody production and atheroprotective antibodies have been identified in both humans and mice[89-91]. However, the results from experimental studies on Th2 cells and atherosclerosis are inconsistent as some studies report decreased atherosclerosis in mice deficient for the Th2 cytokine IL-4, whereas others show no effect[92, 93]. These results indicate that the role of Th2 cells and their related cytokines in atherosclerosis is complex and needs to be further investigated in order to rule out if Th2 cells are a possible target in antiatherosclerotic therapies. A Th2 cell response is associated with B cell activation, plasma cell differentiation and production of antigen specific antibodies. B cells are also found in atherosclerotic lesions[94] and they have generally been considered to be antiatherogenic based on results from experiments with splenectomised mice. Spleen is a major B cell reservoir and splenectomy leads to increased atherosclerosis which can be reversed by B cell replacement[95]. It has also been shown that hypercholesterolemic mice deficient in B cells (via depleting the gene encoding the μ-chain of the BCR, μMT) have more atherosclerosis implicating that B cells are atheroprotective[96]. However, the role of B cells in atherosclerosis is now debated since recent data from two independent groups shows that blocking B cells with an antibody decreases atherosclerosis development in mice[97,98,99]. The atheroprotective role of B cells is also based on the fact that IgM and IgG have been shown to reduce atherosclerosis. Anti-oxLDL IgM antibodies are associated with atheroprotection, possibly through their capacity to bind oxLDL and thereby inhibit their uptake by macrophages and prevent foam cell formation[100]. Moreover, intravenous administration of polyclonal IgG antibodies reduces plaque formation in hypercholesterolemic mice[91], possibly via activation of the complement system[100], and clinical studies have shown an association between high levels of IgG autoantibodies to apoB-100 peptides and less coronary atherosclerosis[90, 101]. Clinical trials with recombinant antibodies specific for an apoB-100 derived peptide are going on at the moment and may result in the first immune modulating therapy against atherosclerosis.

NKT cells – the involvement of lipid antigens in atherosclerosis

NKT cells are innate-like T cells that recognize lipid-antigens presented on CD1. As mentioned above, oxLDL is an important antigen in atherosclerosis, and the fact that oxLDL to a large extent is lipid containing further implicates the role of lipid antigen presentation and NKT cell activation in atherosclerosis. Several CD1 expressing APCs have been identified in atherosclerotic plaques suggesting that NKT cell activation takes place there[102]. Experimental studies have shown that activation of NKT cells aggravates atherosclerosis whereas mice deficient in CD1d have reduced lesion size[103,104]. NKT cells secrete both pro- and anti-inflammatory cytokines but their role in atherosclerosis is believed to be proatherogenic.

Tregs - atheroprotective immunity

Tregs are immune inhibitory cells that play an important role in maintaining self-tolerance. There are several different subsets of Tregs; natural CD4CD25Foxp3 expressing Tregs (nTregs) and the inducible Tregs (iTregs) that secrete IL-10 (Tr1 cells) or TGF-β (Th3 cells)[105]. Deficiency of nTregs results in severe autoimmunity[48]. Studies from hypercholesterolemic mice have shown that atherosclerosis is increased when nTregs are depleted[106] and mice with increased number of nTregs have less disease[107]. Thus, nTregs are believed to protect against atherosclerosis by controlling the proinflammatory activity of other T cells specific for modified self-antigens in the vascular wall. Atherosclerotic lesions contain very few Tregs as opposed to normal arterial tissue[108]. This suggests that the local tolerance is impaired in the plaques. Tregs induce immunosuppression both through cell-cell contact-dependent mechanisms and through secretion of the anti-inflammatory cytokines IL-10 and TGF-β. Hypercholesterolemic mice deficient in IL-10 have increased atherosclerosis with more macrophages infiltration in lesions, increased Th1 cell response and less accumulation in plaques[109,110]. It has also been shown that experimental atherosclerosis can be reduced when a Tr1 response is induced via immunizations[111]. Hypercholesterolemic mice with neutralized TGF-β have increased and more advanced atherosclerosis[112]. These results have been confirmed in a mouse model were TGF-β signaling in T cells was disrupted which resulted in severe atherosclerosis associated with increased T cell and macrophage infiltration and reduced collagen content further indicating that TGF-β has atheroprotective properties[113]. In conclusion, the experimental studies on atherosclerosis and Tregs consistently show that Tregs are atheroprotective. However, the role of human Tregs in atherosclerosis is not fully elucidated and the number of clinical studies on Tregs and atherosclerotic disease is limited. Specific activation of Tregs as a potential target in new immune modulating therapies needs to be further investigated.

The role of costimulation and coinhibition in atherosclerosis

CD4+ T cell activation requires a combination of two different signals from the APC. Signal 1 is the antigen presentation on MHC class II and signal 2 is the costimulatory molecules that are expressed on the APC surface and bind to receptors on the T cell. Costimulatory molecules are expressed on fully activated APCs. Coinhibitory molecules or the lack of costimulatory signals may lead to inactivation of T cells. Presentation of self-derived antigens is associated with absence of costimulation to protect the host from autoimmune responses. Several costimulatory molecules have been shown to play a role in atherosclerosis development and modulation of the expression of costimulatory or coinhibitory molecules could be a potential strategy to direct an immune response into a more antiatherogenic response[17,114,115]. An overview of the role of cell costimulatory and coinhibitory pathways in atherosclerosis is presented in Table 1.

Table 1. T cell costimulation and coinhibition in atherosclerosis

Receptor and Ligand	Animal model	Effect on atherosclerosis
CD28 and CD80/CD86	CD80/86$^{-/-}$LDLR$^{-/-}$	Decreased[116]
CD28 and CD80/CD86	CD28$^{-/-}$ BMT or CD80/86$^{-/-}$ BMT into LDLR$^{-/-}$	Increased[106]
ICOS and ICOS ligand	Generation of anti-ICOS antibodies in ApoE$^{-/-}$	Increased[117]
ICOS and ICOS ligand	ICOS$^{-/-}$ BMT into LDLR$^{-/-}$	Increased[118]
OX40 and OX40 ligand	OX40 ligand$^{-/-}$ in C3H/He mice	Decreased[119]
OX40 and OX40 ligand	Neutralizing OX40 ligand with antibody	Decreased[114]
CD40 and CD40 ligand	Antibodies neutralizing CD40 ligand in LDLR$^{-/-}$ mice	Decreased[120]
CD40 and CD40 ligand	CD40$^{-/-}$ApoE$^{-/-}$ mice	Decreased[121]

Atherosclerosis from a clinical perspective

During atherosclerosis development the artery wall will become thicker. The thickening will be compensated for by gradual dilation to keep the lumen diameter unaltered. However, as the atherosclerotic lesion enlarges it will at some point intrude into the lumen and alter the blood flow[63]. This might cause clinical complications such as angina pectoris and claudication intermittens. Atherosclerosis becomes a life threatening disease when blood is exposed to the pro-thrombotic material inside the plaque, either as a consequence of plaque rupture or erosion, leading to thrombus formation. If the thrombus detaches and is carried away by the blood stream it is called an embolus. A thrombus or embolus will cause an occluded artery resulting in ischemia, leading to an infarction of the tissue normally supplied by the vessel. If the occlusion is in a coronary artery, the occlusion will lead to a myocardial infarction, usually called heart attack. If the occluded artery is located in the brain, it causes an ischemic stroke[122]. Atherosclerosis is a "silent disease" meaning that many people have atherosclerotic plaques without any symptoms. Thus, myocardial infarction or stroke often occurs without any previous warning signs. The atherosclerotic burden increases with age and as people are getting older the incidence of CVD increases. However, the mortality from CVD has decreased the last 30 years due to better prevention and better care[62].

The clinical manifestations of atherosclerosis include angina pectoris, myocardial infarction, transient ischemic attack (TIA), ischemic stroke and claudication intermittens (claudication). Angina pectoris is a temporary pain in the chest caused by an atherosclerotic narrowing of a coronary artery. Angina pectoris often occurs when the oxygen demand in the body is increased, e.g. at exercise or emotional stress. Stable angina pectoris rarely causes any damage to the myocardium. Ischemia in myocardial infarction differs from angina pectoris in the way that its' duration is longer so it results in tissue destruction. Classical symptoms of a myocardial infarction are intensive chest pain, shortness of breath, sweating, nausea and fatigue. The symptoms differ sometimes between men and women as women often have more unspecific symptoms. Myocardial infarction is diagnosed with electrocardiography and biochemical markers in blood. The treatment of a myocardial infarction is based on several different strategies: Pain is reduced with morphine; ischemia is prevented with drugs (betablockers and nitroglycerin) and/or invasive revascularization. Defibrillation is used in cases of arrhythmias and thrombolytic drugs are given to clear the blood clot and prevent new thrombus formation. Heart failure often follows a myocardial infarction and diuretics and angiotensin-converting enzyme (ACE) inhibitors are used to reduce the symptoms of heart failure. TIA and ischemic stroke are caused by atherosclerotic disease in the carotid and cerebrovascular arteries. TIA and ischemic stroke are defined in the same way with the difference that a TIA is transient (thereby

the name) in contrast to an ischemic stroke where symptoms prevail for at least 24 hours. The symptoms of cerebrovascular ischemic events start rapidly and reflect the area of the brain that is affected. Paralysis, vision loss, aphasia and changes in perception are classical symptoms of a TIA or ischemic stroke. In case of an ischemic stroke, thrombolytic treatment should be started as soon as possible to reduce the area of damaged neuronal tissue[122, 123].

Antiatherogenic drugs

Prevention of atherosclerosis is either aiming at the risk factors or directly at the diseased artery. Primary prevention is aiming at decreasing the risk of a first cardiovascular event and includes both lifestyle changes and treatment with drugs, most commonly lipid lowering statins. The aim of secondary prevention is to lower the risk of a second cardiovascular event. The overall aim of both primary and secondary prevention is to slow down the atherosclerotic process in the vascular wall[122].

Statins

Statins are by far the most common group of lipid lowering drugs. They bind to and inhibit 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase, the rate limiting enzyme in cholesterol synthesis leading to a reduced generation of cholesterol in the liver. In response to the reduced cholesterol synthesis there is an increased expression of hepatic LDL-receptors resulting in increased uptake of LDL from plasma. Statin treatment can also reduce triglyceride levels and increase HDL cholesterol in addition to its effect on plasma LDL[124]. Statins are used in both primary and secondary prevention of cardiovascular diseases and several large clinical trials have been performed to evaluate the effect of statins on mortality and morbidity in CVD. The Scandinavian Simvastatin Survival Study (abbreviated 4S) evaluated the effect of the statin simvastatin in patients with manifest coronary heart disease. The 4S study showed a ~30% reduction in the risk of death in patients treated with simvastatin compared to placebo[125]. The aim of the JUPITER trial was to evaluate whether statins reduce the risk of myocardial infarction or strokes in persons with normal LDL cholesterol levels but with increased C-reactive protein (CRP). The results showed that rosuvastatin reduced the risk of cardiovascular events in persons with normal LDL cholesterol[126]. Some statins have also the potential to induce regression of manifest atherosclerotic disease[127]. In addition to its lipid-lowering effect, statin treatment is associated with anti-inflammatory effects such as reduction in CRP and reduced activation of leukocytes[128]. The impact of the anti-inflammatory properties of statins to reduce CVD need to be further investigated. Statins are usually well tolerated and side effects are uncommon.

Methods

This method section is only an overview of some of the methods used in this thesis. It will also provide a discussion about the advantages and disadvantages of the specific methods. Please see the individual Paper I-VI for detailed material and method descriptions.

Mouse models of atherosclerosis

Mice are the most common animals used in atherosclerosis research because of their small size and relatively short life span. However, most mouse strains are resistant to atherosclerotic lesion development even when fed a diet with high fat and cholesterol content. The C57BL/6 mouse strain is the most atherosclerosis sensitive and therefore also the most used model in experimental studies of the disease. C57BL/6 mice are prone to Th1 immune responses in contrast to other mouse strains e.g. BALB/c that are Th2 prone[88]. To develop severe atherosclerosis, C57BL/6 mice need to be genetically modified to disturb the normal lipid metabolism. By feeding the mice a diet with high fat and cholesterol content (termed high fat diet, HFD), atherosclerosis development can be further increased. Normal mouse chow diet contain 4-6% (w/w) fat and <0.02% cholesterol. The high fat and cholesterol containing diet used in the studies in this thesis contains 21% fat (cocoa butter fat) and 0.15% cholesterol. By deleting either the LDL receptor ($LDLR^{-/-}$) gene or the ApoE ($ApoE^{-/-}$, one of the ligands for the LDLR) gene the mice will develop increased cholesterol and triglyceride levels in plasma, which is further increased by HFD. The LDLR is a cell surface receptor recognizing apoB-100 and ApoE in LDL and VLDL, respectively and both LDLR and ApoE are important in clearance of LDL particles. $LDLR^{-/-}$ mice need to be fed a HFD to develop severe atherosclerosis as opposed to $ApoE^{-/-}$ mice that develop lesions even on normal mouse chow diet[129]. Apobec1 is the enzyme responsible for inducing a hypermutation in apoB mRNA to produce the apoB-48 rather than the full-length apoB-100 protein. apoB-48 is synthesized in the intestine and is also required for lipoprotein metabolism in the intestine [130]. Mice deficient for apobec1 can only produce the full length apoB-100 and mice deficient in both apobec1 ($apobec1^{-/-}$) and LDLR are more atherosclerosis-susceptible than the $LDLR^{-/-}$ model and develops atherosclerosis even when fed a chow diet. The mouse models of

atherosclerosis that we use in our laboratory are primarily ApoE$^{-/-}$ (from Taconic or Jackson Laboratory) or the LDLR$^{-/-}$apoBec1$^{-/-}$ (originally from Jackson Laboratory). We normally start feeding our mice HFD from 8-12 weeks of age until termination of the experiment, usually at ~25 weeks of age. Despite the relatively long period on HFD our mice develop relatively small lesions, both in the aortic root and in the descending aorta, compared to what is seen from other groups using similar conditions. One can only speculate for what reason that is, but it could be due to the extremely pathogen-free conditions in our animal facility. Our mice are exposed to very few pathogens compared to mice in wild life and even in comparison with most other animal houses that are having other pathogen conditions. The pathogen-free conditions induce less immunological activity in the mice. The fact that our mice have rather small lesions sometimes complicates comparisons were relatively small differences in lesion size are expected[131]. On the other hand, the extremely pathogen-free conditions in our animal facility allow us to breed immunodeficient mice under standard conditions without any side effects. ApoE$^{-/-}$ mice on C57BL/6 background develops lesions throughout the whole arterial tree. The process involves fatty streaks, foam cell formation and finally fibrous plaques. The process is accelerated by feeding the mice HFD and those mice will have more lipid-rich lesions compared to chow fed animals[132]. The morphology of the murine atherosclerotic plaques shares several similarities with human plaques. The plaques have a necrotic core and a fibrous cap containing smooth muscle cells, collagen fibers and elastic matrix proteins. However, atherosclerotic complications such as plaque rupture and thrombosis is very unusual in mice[133]. The plasma cholesterol levels of ApoE$^{-/-}$ mice fed HFD are relatively high (~20-25 mmol/L in our hands) compared to in humans, even without statin treatment (~4-6 mmol/L).

Mouse models used in this thesis

ApoE$^{-/-}$ mice were used in Paper I, II and IV. Moreover, C57BL/6 was used as a control in Paper I and in Paper IV were ApoE$^{-/-}$ mice crossed with MHC class II deficient (MHCII$^{-/-}$) mice (from Jackson Laboratory) in order to obtain the double knock-out ApoE$^{-/-}$MHCII$^{-/-}$ mouse model. In Paper III we used LDLR$^{-/-}$ mice that were bone marrow transplanted with bone marrow deficient for FcγRIIB. Transplantation of bone marrow from C57BL/6 mice served as control. To enhance atherosclerosis development they were fed HFD (Paper I and II from 10 weeks of age, Paper III from 16 weeks of age and in Paper IV from 8 weeks of age) until the experiments were terminated at week 24 (Paper III) or 25 (Paper I, II and IV). In Paper V we used C57BL/6 mice deficient for CD1d (CD1d$^{-/-}$), their heterozygous littermates (CD1d+/-) and normal C57BL/6 mice were used as controls. In Paper V we induced neointima formation in response to mechanical injury with a carotid

collar model[134] and the mice were fed normal mouse chow diet throughout the experiment.

Evaluation of experimental atherosclerosis

Atherosclerosis in experimental mouse models is assessed in the aorta and/or in the aortic root. Atherosclerosis develops earlier in the aortic root compared to the aorta [129]. Atherosclerosis in the aorta is assessed by *en face* preparations of the vessel stained with the neutral lipid stain Oil-red-O (ORO) [135]. Stained plaque areas were quantified blindly using Image ProPlus 4.5 software and plaque size is expressed as ORO stained area in percent of total surface area. For evaluation of atherosclerosis in the aortic root, immunohistological analysis was made on 10 μm cryo-sections. Sections were stained with hematoxylin to facilitate orientation and determination of the lesion borders. To detect monocytes/macrophages sections were stained with anti-mouse MOMA-2 antibodies and anti-human CD3 (cross-reactive with mouse) were used to determine T cell content. The sections were counterstained with hematoxylin to detect cell nuclei. Quantification of MOMA-2 or CD3 stained areas were conducted blindly using the BioPix iQ 2.0.16 software and the MOMA-2 or CD3 stained area were expressed as percentage of the total area. The total plaque area was also determined using BioPix iQ. Quantification of immuno-histological staining as well as ORO stained aortas were always conducted blindly. The same person performed all the measurements within a project as the technique is operator sensitive.

Peptide immunization

In Paper II we used the apoB-100 derived peptide p210 coupled to cationized bovine serum albumin (cBSA) together with Alum as adjuvant (aBp210) in a 1:1 ratio. In paper I Alum was used in immunizations together with PBS but without adding any antigen. According to the protocol from the manufacturer (Pierce), Alum was added drop wise during mixing with PBS or antigen using a magnetic stirrer. Alum and PBS or aBp210 were subsequently allowed to be mixed for 30 minutes. $ApoE^{-/-}$ mice were then immunized subcutaneously in the neck with 100 μl Alum and PBS (Paper I) or aBp210 (Paper II). Immunizations were carried out after a brief sedation with isoflurane in order to immobilize the animals. Mice were immunized at 6, 9 and 11 weeks of age. A third booster was given at week 24 to increase the possibility to detect a treatment-specific immune response.

Neutralizing CD25 antibodies

Neutralizing CD25 antibodies have previously been used to delete nTregs which express high levels of CD25 (the α chain of the IL-2 receptor) on their surface[106, 136]. To determine the role of CD25 in the immune response to aBp210 we gave weekly intra-peritoneal injections of 100 μg neutralizing CD25 antibody from week 6 of age (at the same time as the first immunization) until week 11, as well as one single injection week 24. nTregs in mice are defined based on their expression of CD4, CD25 and Foxp3. In mice, the majority of the CD4+ cells expressing CD25 are also expressing Foxp3. However, there is a small population of CD4+CD25+ cells that are not Tregs, but probably representing a T effector cell population. This small population of CD4+CD25+ T effector cells is also depleted by the neutralizing CD25 antibodies. Consequently, when interpreting the results from these studies it is important to keep in mind that not only Tregs are depleted and that the effect observed may be due to the deletion of other cells than Tregs.

Proliferation assay

Proliferation of cultured splenocytes in response to polyclonal activation was used in Paper I-IV. Splenocytes in single cell suspension were cultured with the polyclonal T cell activators concanavalin A (ConA) or CD3/CD28 beads (Invitrogen). Non-treated cells were used as controls. After 72 hours of culture ^{3}H-Thymidine was added and the cells were cultured for an additional 16 hours. The amount of ^{3}H-Thymidine incorporated into the cells was determined with a liquid scintillation counter that measures the energy emitted by the radioactive substance. The amount of emission is given as counts per minute (cpm) and the rate of proliferation was presented as a Proliferation Index that is cpm from stimulated cells divided by CPM from non-stimulated cells. In Paper I-IV we measured the Proliferation Index in order to examine the activation state of the T cells derived from spleen. The proliferation rate mirrors the present activation state of the T cells and we interpret the results as if there is a high Proliferation Index the T cells are prone to proliferate and are mainly effector cells. If the Proliferation Index is reduced we interpret the result as there is an increased suppressive activity among the T cells.

Flow cytometry

With flow cytometry it is possible to detect properties of individual particles in a sample. In the studies included in this thesis, flow cytometry has been used to detect

and characterize populations of immune cells from spleen or blood samples. We have used a CyAn ADP flow cytometer from Beckman Coulter. CyAn ADP has three lasers that emit light of different wave lengths and there are nine different detectors that can distinguish emission from up to nine different fluorescent compounds. The flow cytometer order the cells into a single cell stream that passes through the lasers. The forward scatter channel (FS or FSC) is a measurement of light that is scattered in the forward direction and reflects the size of the cell. The side scatter channel (SS or SSC) provides information about the granularity of the cell and the combined FS and SS can be used to roughly differentiate between cell populations. Since specific populations of immune cells express their own individual pattern of molecules, flow cytometry can be used to identify these populations. Fluorochrome-labeled antibodies directed to cell surface receptors or intracellular molecules are used to stain cells prior to flow cytometric analysis. When two or more fluorochrome-labeled antibodies are used within the same analysis there is a possibility that the fluorochrome emissions will coincide. Some fluorochromes give rise to a strong signal through a specific detector, whereas others give rise to a weak signal in two or more detectors. Thus, with increasing amounts of fluorochrome-labeled antibodies the risk of spectral overlap increases. This spectral overlap will make measurement of the true fluorescence difficult. Therefore, during data analysis, a process called fluorescence compensation is applied to calculate the interference between different fluorescence detecting channels. Gating is the procedure when specific populations of cells are selected. To simplify gating, single stained control cells or fluorescence minus one (FMO) are used. Single stained controls are cells that are stained with only one of the fluorochrome-labeled antibodies used in the experiment. Fluorescence minus one (FMO) are samples stained with all fluorochrome-labeled antibodies in the experiments, except one. Non-stained cells are used as a negative control. With single stain controls or FMO it is possible to determine where the positive cell populations should be plotted. Despite use of both a single stained control and FMO to avoid errors in the analysis, flow cytometry analysis of cells stained with multiple fluorochrome-labeled antibodies is complex. Experience with the different fluorochrome-labeled antibodies and knowledge about the pattern they give in different cell types increases the safety of the analysis. Proper handling and maintenance of the instrument is also crucial to acquire reliable results from flow cytometry.

Staining procedure

We incubated whole blood or splenocytes in single cell suspension with fluorochrome-labeled antibodies. One million splenocytes or 20 µl of blood were first incubated with antibodies targeting the Fc receptor in order to block the Fc part of

antibodies already bound to the cell. This will reduce unspecific binding of the fluorochrome-labeled antibodies used in the experiment. The mixture of fluorochrome-labeled antibodies (from Biolegend or eBioscience) specific for each experiment was then added to the cells. After 30 minutes of incubation on ice, the cells were washed twice with PBS supplemented with 1% fetal calf serum and 0.5 mM EDTA (FACS-buffer). If labeling cells in blood, erythrocytes were lysed after the 30 minutes incubation and then washed three times with FACS-buffer. Cells were then fixed and permeabilized with a Fix/Perm kit from eBioscience. In some studies fluorochrome-labeled antibodies directed against intracellular antigens were thereafter added and cells were incubated for another 30 minutes on ice. Finally, the cells were washed and resuspended in FACS-buffer.

Malmö Diet and Cancer Study

Between 1991 and 1996, about 17000 females and 11000 males aged 45-64 years were included in the Malmö Diet and Cancer Study (MDCS) to generate a population cohort for investigating the association between diet and cancer. More than 6000 subjects were also invited to take part in a sub-study focusing on cardiovascular risk (the cardiovascular arm of MDCS). Blood was collected and mononuclear leukocytes were isolated from these subject and the cells were frozen and stored at -140^{o}C. The plasma and serum from these subjects were stored at -80^{o}C. At baseline, information about food intake, life-style patterns, heredity, profession, previous and current diseases and medications was obtained. Intima media thickness (IMT) of the carotid arteries, which reflects the atherosclerotic lesion size, was assessed by B-mode ultrasound. The participants have been followed to monitor incidence of myocardial infarction and stroke. We randomly selected 700 subjects from the cardiovascular cohort of MDCS to study if there was an association of circulation Tregs and the incidence of a cardiovascular event or carotid IMT. A cardiovascular event was defined as sudden cardiac death, fatal or nonfatal myocardial infarction, fatal or nonfatal stroke or death because of underlying CVD, whichever came first. The mononuclear leukocytes that were stored at -140 ^{o}C for more than 15 years were thawed and cells were stained with fluorochrome-labeled antibodies to detect Tregs with flow cytometry. The viability of the cells when thawed were >90% as assessed with 7AAD viability stain. Tregs were defined based on their expression of CD4, CD25 and Foxp3. We also applied a second definition of Tregs based on the expression of CD4 and CD25 since Foxp3 is not constitutively expressed on human Tregs. To characterize the functional properties of the mononuclear cells from the subjects, cells were cultured for 72 hours in presence of CD3/CD28 beads to induce polyclonal activation of the cells. Th1, Th2 and Treg specific cytokines in the cell supernatants were analyzed with multiplex technology (MesoScale Discovery) to

determine the immunological profile of the cells. Flow cytometric analysis of all the 700 subjects included in this study was carried out during a time period of almost one year. To be able to perform this kind of large study with stable and reliable results it requires very technically skilled persons to perform it as well as accurate maintenance of the instrument.

Ethics

All animal experiments included in this thesis followed Swedish guidelines for care and usage of experimental animals. The Malmö/Lund regional ethical committee for laboratory animals approved the animal experiments performed. The clinical studies were approved by the Ethics Committee of the University of Lund and were conducted in accordance with the Helsinki Declaration. All subjects gave written consent.

Present investigation

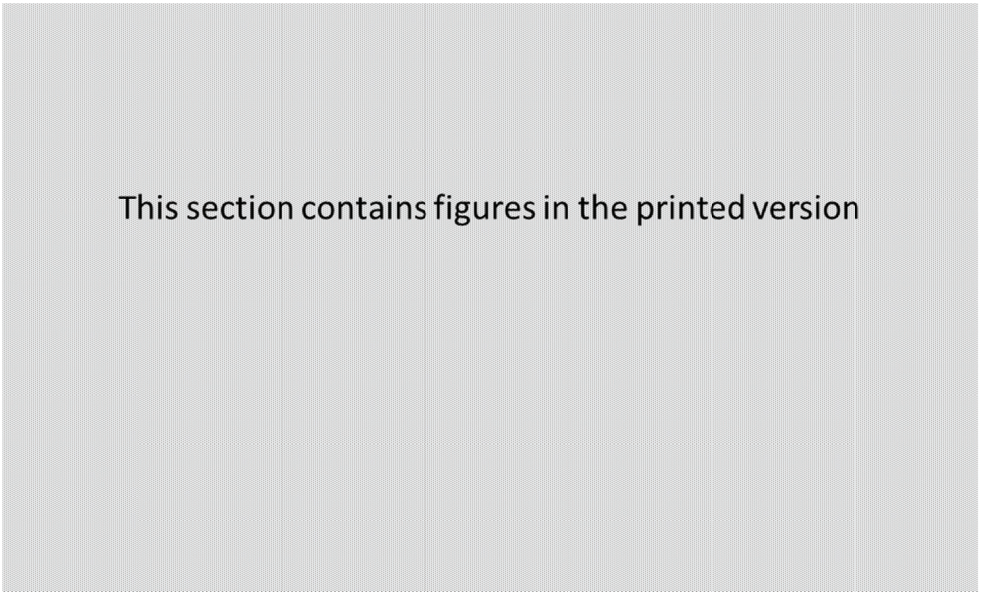
The objective of this thesis was to study atheroprotective immune responses, either induced by immunization or naturally occurring in the process of atherosclerosis development. The general aim of the research in our group is to develop immune modulating therapies to induce protective immune responses in atherosclerosis. An additional aim is to find biomarkers to predict the risk of future cardiovascular disease. Earlier studies from our group have shown that immunizing hypercholesterolemic mice with apoB-100 related peptides combined with Alum is associated with a reduction in atherosclerosis development by up to 60%. However, during these studies two major questions have been raised; (1) how is the protective immune response induced and (2) why is Alum, without any antigen, also inducing atheroprotection? These two questions have been addressed in Paper I and II. In Paper I we found that Alum can induce atheroprotection in hypercholesterolemic mice and that this most likely is explained by a local uptake of antigens from LDL at the injection site. In Paper II we showed that immunization with the apoB-100 related peptide p210 together with Alum induces a protective immune response with increased presence of Tregs. The protective effect of aBp210 was inhibited when mice were treated simultaneously with antibodies neutralizing Tregs.

To be able to further improve the formulation of a future vaccine against atherosclerosis, we also studied the role of several different components of the immune response to identify important pathways in atherosclerosis development. In Paper III it was shown that the inhibitory FcγRIIB has a protective role in atherosclerosis. This suggests that aiming at an increased binding of antibodies and immune complexes to FcγRIIB could be a potential way to induce atheroprotection. In Paper IV the role of antigen presentation on MHC class II in atherosclerosis development was studied. Hypercholesterolemic mice with MHC class II deficiency had increased atherosclerosis development indicating that antigen presentation on MHC class II contributes to the activation of protective immunity in the disease. In Paper V, the role of CD1d, the molecule presenting lipid antigens to NKT cells was studied using a model of vascular injury. The results showed that neointima formation was reduced in mice deficient for CD1d, suggesting a role for NKT cells and lipid antigens in the vascular repair processes. Such repair processes are believed to play a key role also in the development of atherosclerosis. As mentioned above, an

additional aim of our research is to find biomarkers that predict the risk of future cardiovascular disease. Therefore in Paper VI, we analyzed circulating Tregs from 700 subjects randomly selected from the cardiovascular arm of the Malmö Diet and Cancer study to evaluate if circulating levels of these cells can predict future cardiovascular events.

Paper I

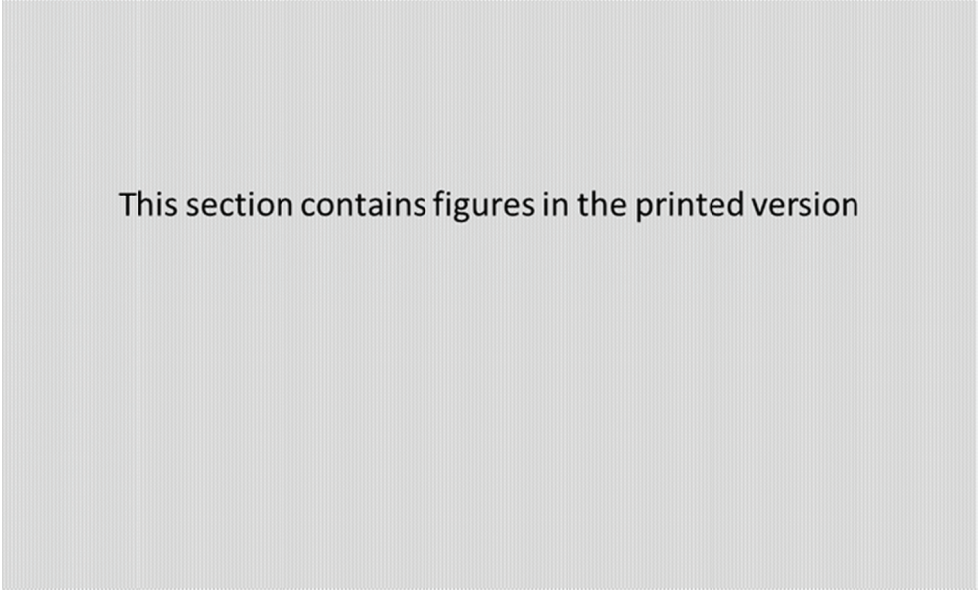
Adjuvants are used to enhance the immunogenicity of the antigen in a vaccine and the specific adjuvant plays a critical role in modulating the immune response that follows vaccination. Aluminum-containing adjuvants are the most widely used adjuvants in human vaccines and they are believed to primarily induce humoral immunity. The mechanisms by which aluminum-containing adjuvants act involve delayed clearance of the antigen and induction of a local inflammation at the injection site which promotes activation of APCs. It has previously been shown that the aluminum hydroxide adjuvant Alum has atheroprotective properties[137] which make Alum a suitable adjuvant in vaccines aiming at decreasing the development and progression of atherosclerosis. The aim of the study in Paper I was to elucidate how atheroprotection is induced by Alum immunization in absence of administration of an antigen. First, we compared the immune response in $ApoE^{-/-}$ and WT (C57BL/6) mice that were immunized with Alum and PBS or with PBS alone. In WT mice, there were no differences in the population size of CD4+ Tregs or effector cells after immunization. However, in $ApoE^{-/-}$ mice, Alum immunization increased the frequency of Tregs (Figure 4A) and reduced the effector CD4+ T cell population. The release of the proinflammatory cytokines IFN-γ and TNF-α from cultured splenocytes stimulated with ConA were increased only in the WT Alum group. These results demonstrated that in $ApoE^{-/-}$ mice, Alum immunization induces a suppressive immune response with an increase in Tregs as opposed to WT mice in which a modest proinflammatory response was induced. Further analysis of the differences between $ApoE^{-/-}$ and WT mice showed that in $ApoE^{-/-}$ mice, but not in WT mice, there was an accumulation of LDL in the subcutaneous tissue. When analyzing the subcutaneous Alum precipitate at 15 minutes or 7 days after injection we found presence of MDA-modified and oxidized LDL. These results suggest that peptides derived from oxidized LDL are captured by the Alum precipitate at the injection site and act as vaccine antigens. Consequently, Alum immunization in $ApoE^{-/-}$ mice, but not in WT mice, mimics immunization where oxLDL or apoB-100 peptides are used as antigens with the aim to reduce atherosclerosis development. In order to study the effect of Alum immunization on atherosclerosis development we immunized $ApoE^{-/-}$ mice with Alum or PBS and fed them a diet rich in fat and cholesterol. When mice were 25 weeks old we assessed atherosclerosis in the descending aorta and the



immunological profile in the spleens of the animals. Alum immunization reduced atherosclerosis development in descending aorta by 35% (Figure 4B). In the subvalvular region, the plaque size did not differ, but the CD3 stain was decreased and monocyte/macrophages were increased in Alum immunized mice compared to the PBS control. Alum immunized mice had increased total cholesterol levels despite a reduction in atherosclerosis in the aorta. The frequency of Tregs was increased and the CD4+CD28+ population was reduced in mice immunized with Alum compared to PBS, which is consistent with the suppressive effect of Alum from the study with younger mice. In conclusion, the results in Paper I indicate that Alum immunization is associated with an increased frequency of Tregs and reduced expression of activation markers on CD4+ T cells in $ApoE^{-/-}$ but not in WT mice. Furthermore, Alum immunization reduces atherosclerosis development in $ApoE^{-/-}$ mice, potentially through capture of LDL related antigens at the site of immunization which initiates a tolerogenic immune response.

Paper II

Previous studies from our group have shown that atherosclerosis development can be inhibited by immunization with the apoB-100 related peptide p210 together with Alum as adjuvant (called aBp210). The immunological mechanism through which atheroprotection is induced has not been characterized before. In Paper II we tested the hypothesis that Tregs are induced by aBp210 and as a tool to block the function of Tregs in $ApoE^{-/-}$ mice we used neutralizing anti-CD25 antibodies. $ApoE^{-/-}$ mice immunized with aBp210 had an increased frequency of Tregs in blood one week after the second booster (12 weeks of age) and in the spleen at week 25 compared to control mice receiving PBS only. To test the suppressive function of the Tregs, spleen T cells were polyclonally activated and the proliferative response determined. Spleen

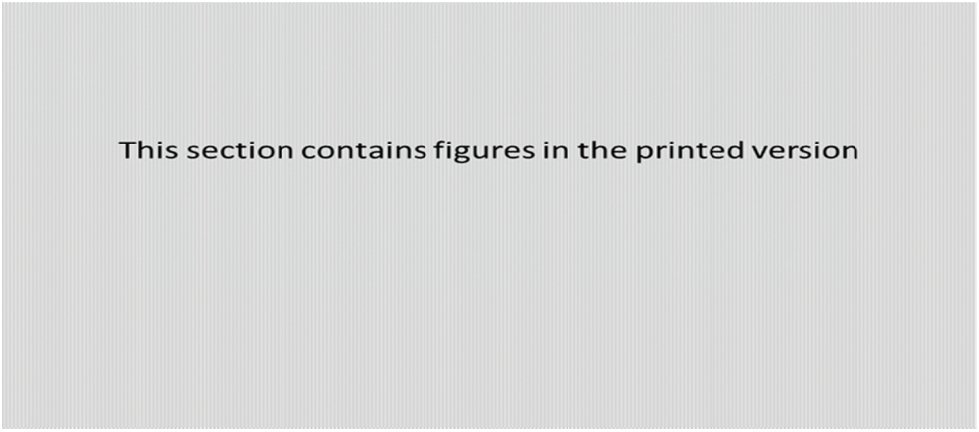


cells from mice immunized with aBp210 proliferated with a lower rate compared to cells from control mice indicating that the increased Treg population had suppressive function. Atherosclerosis in the descending aorta was reduced by 37% in mice immunized with aBp210. To further investigate the role of Tregs in the immune response induced by aBp210 we gave mice weekly intraperitoneal injections of neutralizing anti-CD25 antibodies during the immunization period. Anti-CD25 antibodies reduced the presence of Tregs in blood at week 12 and in spleen at week 25. Mice receiving both aBp210 and anti-CD25 antibodies did not have the same reduction in atherosclerosis development that was seen in aBp210-immunized mice (Figure 5). However, the suppressive effect in the proliferation assay was still present which may be due to a sustained binding of the anti-CD25 antibodies to effector T cells. Immunization with aBp210 was associated with an increased secretion of IL-10

from polyclonally activated splenocytes indicating that there is an activation of Tregs after aBp210 immunization. IL-5 was increased after aBp210 immunization both in plasma and in activated cultured splenocytes indicating that a Th2 response also is induced. When using neutralizing anti-CD25 antibodies there was an increase in Th1, Th2 and Treg-related cytokines in plasma. Taken together, the results in Paper II showed that Tregs may have a role in the protective immune response induced by aBp210 immunization.

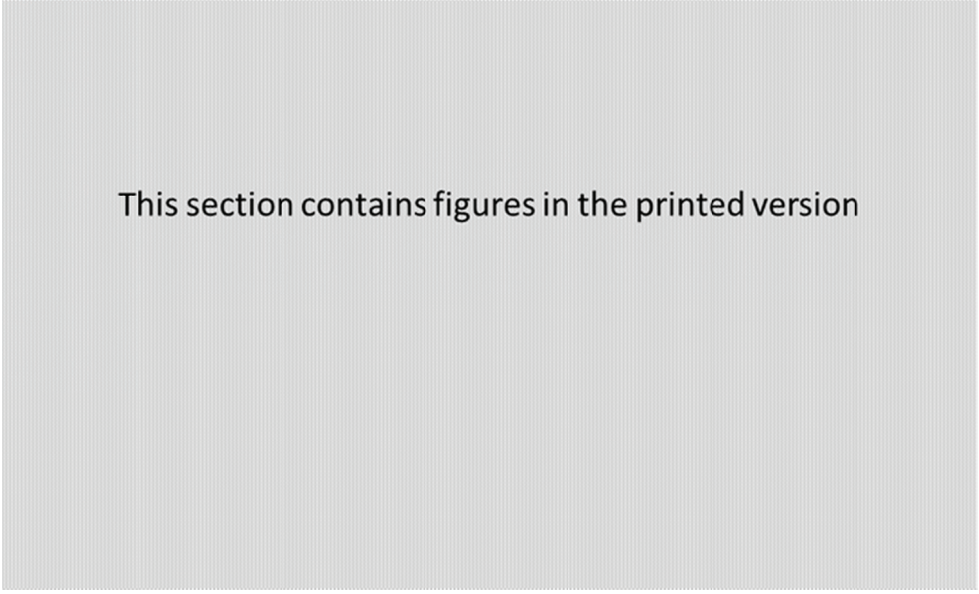
Paper III

In Paper III we studied the role of FcγRIIB in atherosclerosis development. FcγRIIB is an inhibitory Fc receptor which means that binding of the Fc part of an antibody to the FcγRIIB leads to a dampened/inhibited activity of the cell expressing the receptor. To obtain hypercholesterolemic mice deficient in FcγRIIB we bone marrow transplanted (BMT) irradiated LDLR$^{-/-}$ mice with FcγRIIB$^{-/-}$ bone marrow. WT bone marrow transplantation served as the control. Paper III shows that deficiency of FcγRIIB induces increased atherosclerosis in the descending aorta compared to WT (Figure 6). FcγRIIB$^{-/-}$ BMT mice had increased fraction of B cells, more activated CD4+ T cells and less Tregs compared to WT BMT mice. Furthermore, the T cells proliferated with a higher rate compared to WT BMT mice. The cytokine profile of FcγRIIB$^{-/-}$ BMT mice indicates that primarily Th2 cells are activated. However, the plasma antibody profile indicated activation of a Th1 response. In conclusion, there seems to be an activation of both Th1 and Th2 cells in FcγRIIB$^{-/-}$ BMT mice which is associated with a reduction in Tregs and an increase in atherosclerosis. The atheroprotective effect of FcγRIIB has recently been confirmed by others using a mouse model that are deficient in both ApoE and FcγRIIB[138].



Paper IV

Activation of CD4+ T cells requires presentation of antigens on MHC class II together with costimulatory signals. In atherosclerosis, CD4+ T cells are believed to be proatherogenic but these cells are, however, counterbalanced by suppressive Tregs. A large fraction of Th1 cells in lesions have been shown to be specific for self-derived molecules such as oxLDL and HSP indicating that atherosclerosis has autoimmune characteristics. In Paper IV we aimed to investigate the role of MHC class II antigen presentation in atherosclerosis development. We crossed $ApoE^{-/-}$ mice with $MHCII^{-/-}$ mice to obtain mice deficient in both ApoE and MHC class II ($ApoE^{-/-}MHCII^{-/-}$). Since MHC class II is required for CD4+ T cell development, $ApoE^{-/-}MHCII^{-/-}$ mice also lack CD4+ T cells. Unexpectedly, the $ApoE^{-/-}MHCII^{-/-}$ mice had more than a

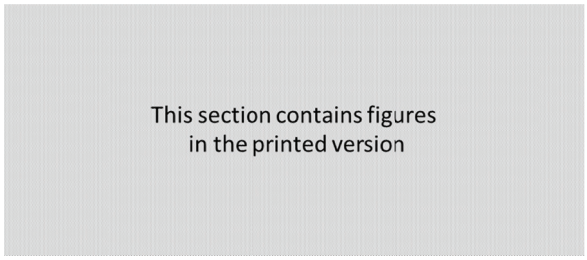


two-fold increase in atherosclerosis in the aorta (Figue 7A) and a 68% increase in plaque size in the subvalvular region (Figue 7B). The increase in atherosclerosis occurred despite a reduction in plasma lipids, body weight, total IgM and IgG and both pro- and anti-inflammatory cytokines. Since $ApoE^{-/-}MHCII^{-/-}$ mice lack CD4+ T cells that are considered to be one of the important disease-promoting cell types in atherosclerosis, other severely proatherogenic factors must operate in these mice. The population of CD8+ T cells was increased by 2.5-fold in $ApoE^{-/-}MHCII^{-/-}$ mice. As opposed to CD4+ T cells, the role of CD8+ T cells in atherosclerosis is not extensively studied and the results are inconclusive. However, it cannot be excluded that the role of CD8+ T cells is altered in mice deficient in both MHC class II and CD4+ T cells. Furthermore, NKT cells have been shown to be proatherogenic and $ApoE^{-/-}MHCII^{-/-}$ mice had more than a 3-fold increase in CD3+NK1.1+ cells. NKT

cells are activated by lipid antigens presented on CD1d and one of the major antigens in atherosclerosis is oxLDL that to a large extent consists of lipids. The increased population of potentially proatherogenic NKT cells may thus be responsible for the increased atherosclerosis development in $ApoE^{-/-}MHCII^{-/-}$ mice. Moreover, the population of protective Tregs is reduced by over 80% in $ApoE^{-/-}MHCII^{-/-}$ mice indicating that these mice lack a small but important regulatory compartment of the immune system (Figure 7C). The reduced number of Tregs was not reflected by an increased systemic inflammation since plasma cytokines were reduced in $ApoE^{-/-}MHCII^{-/-}$ mice. However, the local effect in the arterial wall appeared to be different from the systemic effect since there was an increased accumulation of monocytes/macrophages in the arterial wall of $ApoE^{-/-}MHCII^{-/-}$ mice. The number of CD115+ cell in spleen was reduced but there was a shift of the monocyte population into a more inflammatory phenotype that has the potential to increase atherosclerosis. The accumulative effect of antigen presentation on MHC class II in atherosclerosis development is, according to the results in Paper IV, protective.

Paper V

Vascular repair responses are of importance in plaque growth and maintenance of plaque stability. We used a collar-injury model to study the role of adaptive immunity to lipid antigens in vascular injury-induced repair. Lipid antigens are presented to NKT cells through CD1d molecules on antigen presenting cells. A plastic collar was placed around the right carotid artery on $CD1d^{-/-}$, CD1d+/- and C57Bl/6 wild type mice. Mice were sacrificed at 3 or 21 days after collar placement. Neointima formation was reduced by 60% in CD1d-deficient mice but there were no differences in neointimal macrophage or α-actin content (Figure 8). IgG and IgM were found in the carotid arteries of $CD1d^{-/-}$ and CD1d+/- mice. Cells expressing CD1d in injured control animals were found in the peri-adventitial area and about 25% of macrophages expressed CD1d. To study the role of activated NKT cells in neointima formation WT mice were treated with α-galactosylceramide (α-GalCer, a synthetic antigen that activates NKT cells through binding to CD1d). However, α-GalCer treatment did not influence neointima formation. Wild type mice sacrificed 3 days after collard placement had a reduced level of CD4+ cells and an increased level of NKT cells in the spleen. B cells and CD8+ cells were not affected. These results suggest that NKT cells as well as lipid antigens presented on CD1d are important in neointima formation after vascular injury.



Paper VI

The role of Tregs in experimental atherosclerosis is extensively studied and hypercholesterolemic mice deficient in Tregs, either by neutralization with antibodies or vaccination, have increased atherosclerosis. The role of Tregs in human atherosclerosis and CVD is uncertain and conflicting results have been reported from studies analyzing the association of Tregs with cardiovascular disease. In Paper VI we investigated if the frequency of circulating Tregs can predict development of acute cardiovascular events in humans. Furthermore, we determined if there was an association between the frequency of circulating Tregs and the severity of atherosclerosis in the carotid artery. To address these questions we randomly selected 700 subjects from the cardiovascular arm of the Malmö Diet and Cancer study. Mononuclear leukocytes that were stored at -140°C at the baseline investigation examination were thawed and stained for flow cytometry in order to analyze Tregs. Tregs were defined based on their expression of CD4, CD25 and Foxp3. We also applied a second definition of Tregs based on the expression of CD4 and CD25 since Foxp3 is not constitutively expressed on human Tregs. Values were given as percent of all CD4+ cells or as cell number per µl blood. Cytokine release from CD3/CD28 activated leukocytes was measured to determine associations between the Treg populations and cytokine release. During follow-up, 150 cases of a first cardiovascular event were registered. One hundred and fifty event-free controls matched for age and gender were selected. Cases were found to have a significantly lower percent of CD4+CD25+ cells than controls when controlling for diabetes, blood pressure and LDL/HDL ratio. Furthermore, a significant association between the percent of CD4+CD25+ cells and IMT in the carotid bulb at baseline was found.

Discussion

The results in Paper I showed that atheroprotection can be induced in ApoE$^{-/-}$ mice through immunizations with Alum and this reduction was associated with an increase in Tregs. In Paper II, ApoE$^{-/-}$ mice were vaccinated with aBp210 resulting in a reduction in atherosclerosis development. As in Paper I, this reduction was associated with an increased frequency of Tregs. In these two studies the atheroprotective effect of Alum alone or aBp210 were about equal when compared to PBS treated mice. This fact raises the question whether the apoB-100 peptide p210 had an additional effect in the immunizations compared to Alum alone. Previous papers from our group have demonstrated that apoB-100 immunizations together with Alum more efficiently reduce atherosclerosis development than Alum by itself[3-5, 139]. The atheroprotective effect of Alum alone is well accepted but this effect seems to be more pronounced in the present studies as compared to what have been previously published. The reason why aBp210 does not reduce atherosclerosis more efficiently than Alum alone in the studies presented in Paper I and II remains to be fully explained. In Paper I we describe Alum uptake of modified LDL antigens in a hypercholesterolemic environment as a potential mechanism through which Alum induces atheroprotection. The modified LDL taken up by Alum consists of several different antigens, apoB-100 derived peptides being some of them. Thus, Alum immunizations in hypercholesterolemic mice mimics Alum+apoB-100 peptide immunizations since Alum capture antigens at the site of injection. Consequently, Alum immunization cannot be regarded as a proper control for antigen specificity in studies like this. Furthermore, the role of aBp210 in inducing an atheroprotective immune response needs to be further investigated and the antigen specificity of the Tregs induced by the vaccine should be tested. However, the problem with testing antigen specificity of Tregs lies within the nature of how these cells act, since antigen activation of Tregs does not necessarily lead to proliferation of the cells and therefore the use of traditional proliferation assays is limited. Instead other assays need to be performed to evaluate the antigen specificity of Tregs for example measurements of Treg cytokine mRNA expression and secretion, in response to the specific antigen. Suppression assays with Tregs activated by aBp210 have been tested but so far without consistent results. More studies on the antigen specificity of the Tregs induced both by Alum and by aBp210 immunization are planned to further investigate the mechanism of action of the immune response resulting in atheroprotection. The importance of

additional investigations of the immune response followed by aBp210 immunization is further emphasized by the circumstance that a vaccine containing this peptide is planned to enter into a clinical phase I trial during 2011. When taking this step it will be of critical importance to know the mechanism of action in order to monitor the response of the treatment.

In Paper II it was suggested that atheroprotection induced by aBp210 is dependent on Tregs. This finding has partly been confirmed by others. The apoB-100 peptide p210 have been coupled to cholera toxin B (CTB, p210-CTB) and used for nasal immunizations of $ApoE^{-/-}$ mice. In this study p210-CTB induces a regulatory Tr1 response and reduced atherosclerosis by 35%[140]. These results further strengthen our hypothesis that immunizations with apoB-100 peptide p210 induce Tregs.

A limitation with the neutralizing CD25 antibody strategy used in Paper II is that these antibodies also may neutralize other CD25 expressing T cells than Tregs. However, the fraction of CD25 expressing non-Tregs cells is very low in mice but the relevance of this small population of cells should still be taken into consideration. Activation of CD25-expressing T effector cells by the immunizations cannot be excluded and since proliferation of T effector cells depend on IL-2 signaling this pathway is likely to be blocked by the anti-CD25 antibody. If atheroprotective effector T cells are present they are most likely of the Th2 subset since Th1 cells exclusively seems to be proatherogenic. The production of the Th2-associated cytokine IL-5 is not blocked with the anti-CD25 treatment which indicates that CD25 is not involved in Th2 activation in this study.

The unexpected increase in atherosclerosis of $ApoE^{-/-}MHCII^{-/-}$ mice described in Paper IV can be a result of several different mechanisms. The number of CD8+ T cells is increased in these mice but other studies have suggested that CD8+ T cells play a minor role in atherosclerosis development[141]. However, it cannot be excluded that the function of CD8+ T cells is altered when CD4+ T cells are absent. NKT cells have been shown to have a proatherogenic role and $ApoE^{-/-}MHCII^{-/-}$ mice have increased number of NKT cells[33]. Activation of NKT cells is associated with secretion of large amounts of cytokines but the NKT cells in $ApoE^{-/-}MHCII^{-/-}$ mice do not seem to be activated since there is a reduction in plasma cytokines, both pro- and anti-inflammatory, which argues against a systemic activation of NKT cells. However, the local effect of NKT cells may be different from the systemic and their role in atherogenesis can be enhanced when CD4+ T cells, particularly Tregs, are absent. The reduction in Tregs is yet another possible mechanism that may be responsible for the increased atherosclerosis in $ApoE^{-/-}MHCII^{-/-}$ mice. Tregs are believed to have the

potential to suppress most other immune cells either via cell-cell contact-dependent mechanisms or via secretion of immune-inhibitory cytokines. The deficiency of Tregs is not reflected by a systemic inflammation in the ApoE$^{-/-}$MHCII$^{-/-}$ mice, as it is in mice totally deficient in Tregs but with all other cells intact. However, in inflamed areas, as the atherosclerotic lesions, the deficiency of Tregs may be mirrored as an increased inflammation.

A limitation with the ApoE$^{-/-}$MHCII$^{-/-}$ mice when aiming to study the role of antigen presentation on MHC class II is that these mice are deficient in CD4+ T cells as well. Interpretation of results is complicated by the fact that it is not possible to distinguish whether the result depends on MHC class II or CD4+ T cell deficiency. Reconstitution of CD4+ T cells via transfer is not possible since the CD4+ T cells requires MHC class II to become activated and the role of naïve T cells in any inflammatory condition is probably very small. Furthermore, it is difficult to account for the compensatory mechanisms in an immune system deficient in both MHC class II and CD4+ T cells. Exogenous antigen presentation may possibly be carried out by MHC class I instead and some of the functions of CD4+ T cells may be taken over by the CD8+ T cell population[142].

The mouse models used in Paper III and IV both resulted in increased atherosclerosis compared to their respective control. In Paper III it was shown that absence of the inhibitory FcγRIIB pathway resulted in an increased systemic inflammation with activation of both Th1 and Th2 cells and a reduction in Tregs leading to increased atherosclerosis. In Paper IV it was showed that ApoE$^{-/-}$MHCII$^{-/-}$ mice have reduced Tregs and increased atherosclerosis but without an increased systemic effect although there was an increased local inflammation in the plaques. One possible explanation could be that the FcγRIIB$^{-/-}$ BMT mice still have a normal T cell population in contrast to ApoE$^{-/-}$MHCII$^{-/-}$ mice that lack CD4+ T cells. Several of the inflammatory cytokines that are increased in the FcγRIIB model, but not in the ApoE$^{-/-}$MHCII$^{-/-}$, are produced by or requires T helper cells for their production. Furthermore, FcγRIIB and MHC class II have very diverse roles in the normal course of an immune response. Taken together, these results further emphasis the complex role of the immune system in atherosclerosis development.

In Paper V we used CD1d$^{-/-}$ mice as a model of deficient lipid antigen presentation. However, it is also a model with deficiency in NKT cells. As in Paper IV, the deficiency in the antigen presenting molecule results in deficiency in the respective T cell. Consequently, it is hard to interpret whether we studied a deficiency in antigen presentation or a deficiency in NKT cells. Anyway, the results in Paper V showed that

lipid antigens and CD1d plays an important role in neointima formation after vascular injury. It was also shown that NKT cells were increased in spleen after vascular injury, further indicating that immune responses to lipid antigens are present. The reason why NKT cells activated by the synthetic ligand α-GalCer do not increase neointima formation in the collar model we used a not fully explained. It can be speculated that activation through the TCR on the NKT cells alone is insufficient to induce a significant response. Activation of APCs via antigen recognition may also be required to induce a proper NKT cell response.

In Paper VI we show that low levels of circulating CD4+CD25+ cells are associated with an increased risk of developing an myocardial infarction or a stoke. Although this result suggests the possibility that circulating Tregs may be used to predict the risk of a cardiovascular event the overlap between cases and controls is too large to effectively discriminate an increased cardiovascular risk. Moreover, the definition of Tregs is much more complex in humans compared to mice. Human Tregs do not constitutively express Foxp3 and Tregs are distinguished on several different markers. In Paper VI we defined Tregs as CD4+CD25+ cells or as CD4+CD25+Foxp3+ cells. Human Tregs can also be defined based on the following characteristics: CD4+ CD25high CD127lowCD49d-[143, 144]. The functional difference between these different ways of defining human Tregs needs to be further elucidated. The most important aspect of the present observation is instead that it is the first study to provide evidence that the atheroprotective role of Tregs described in experimental animals is of importance also in humans. In Paper VI we also demonstrate that the frequency of circulating CD4+CD25+ cells is correlated with the IMT in the carotid bulb. This result was unexpected and suggests that Tregs may increase in response to increased atherosclerosis. The increase in Tregs may thus be a way to counterbalance the increased amount of inflammation in the vessels. The results from Paper VI are inconsistent and if Tregs is a marker of the atherosclerotic burden or a predictor of cardiovascular risk needs to be further explained.

Conclusions and future perspective

The atheroprotective role of Tregs in experimental atherosclerosis is well established. Whether Tregs have the same potential in humans needs to be further investigated. However, results from a study included in this thesis suggest that Tregs may have an important role in human atherosclerosis as well. Based on these results, new immune modulating therapies aiming at activating and/or increasing the number of Tregs in order to regress and/or stabilize atherosclerotic lesions, seem to have the potential to be effective. Our mouse studies on immunizations with aBp210 indicate that this peptide vaccine induces Tregs. If this will be the case in humans as well needs to be examined. With increased knowledge of how Tregs are activated it will be possible to improve the formulation of a future aBp210 vaccine to more specifically promote Treg activation. Additional studies on how aBp210 induces atheroprotection and the role of Tregs are ongoing in our laboratory. Furthermore, the antigen specificity of the Tregs needs to be determined. The potential disadvantages with a therapeutic activation of Tregs in order to reduce inflammation in atherosclerotic lesions are that it may result in a general immunosuppression. This disadvantage is more likely to happen if the Tregs not are antigen specific.

Immunization studies with aBp210 on already established atherosclerosis are required to evaluate if aBp210 have the potential to induce regression of the disease. From a clinical view, this will be an important feature of new antiatherosclerotic therapies since the potential to induce plaque regression of the existing therapies is limited.

Studies on the immunological pathways involved in atherosclerosis are important both from a scientific point of view but also when it comes to development of new therapies. If the antiatherogenic role of FcγRIIB seen in mice is applicable also in humans, aiming at enhance binding of IgG to FcγRIIB would also be a potential strategy for new immune modulating therapies against atherosclerosis. Previous studies from our group have shown that passive immunization with monoclonal IgG1 specific for the apoB-100 peptide 45 (p45) can both inhibit development and induce regression of experimental atherosclerosis[145, 146]. These monoclonal antibodies are now in clinical trial and may result in the first immune modulating therapy against atherosclerosis. The atheroprotective mechanism of these antibodies is not fully elucidated but they do not seem to involve Tregs. Both the Fragment antigen binding

(Fab) part and the Fc region of the antibodies seem to be responsible in mediating the atheroprotection as irrelevant antibodies with similar Fc part as the active IgG do not reduce atherosclerosis[146]. On the other hand, mice receiving only the Fab fragment derived from IgG were not protected from atherosclerosis in a model were intact human IgG were used to reduce atherosclerosis[147]. The Fc fragment of antibodies has two important properties that may mediate this effect, binding to FcγR and activation of the complement system. IgG binding to FcγRIIB on B cells inhibits activation and further antibody production by the cell and the atheroprotective effect of the passive immunization may be due to this anti-inflammatory pathway. Furthermore, the Fab fragment of the p45 specific antibodies can possibly mediate atheroprotection through clearance of oxLDL from plasma. p210 specific antibodies were detected in mice immunized with aBp210 which suggests that atherosclerosis can be reduced by activation of several different immunological pathways. If atheroprotection would be enhanced if both activation of Tregs and induction of protective antibodies are combined needs to be investigated.

As previously mentioned, T cell activation is dependent of DC antigen presentation. The mouse models we have used so far in our immunization studies with aBp210 (and other apoB-100 related peptides as well) all have identical MHC molecules. The human HLA molecule is on the other hand very diverse. The properties of the HLA molecules determine which peptides that can be presented by APCs. The diversity in HLA may result in that p210 cannot be properly presented by all APCs since the peptide does not fit into the antigen binding cleft of the HLA molecule. This fact may be a drawback when using the vaccine in humans.

Generally, there is a great need for new biomarkers to predict the risk of myocardial infarction and stroke. Results included in this thesis suggest that Tregs may be a potential biomarker to predict the risk of a future cardiovascular event. However, these results need to be confirmed by others since our study also showed that Tregs may be correlated with the amount of atherosclerosis. New biomarkers may also have implication when it comes to evaluation of the clinical trials with the aBp210 vaccine. It still remains to be determined how to best monitor the effect and efficacy of the vaccine in humans.

The complex role of T cells in atherosclerosis has, if possible, become even more complex when taking our results in the ApoE$^{-/-}$MHCII$^{-/-}$ mouse model into account. CD4+ T cells are generally regarded as proatherogenic[75, 76]. However, the results from the ApoE$^{-/-}$MHCII$^{-/-}$ mouse model show that atherosclerosis is increased in mice deficient in CD4+ T cells. CD4+ T cells are a heterogeneous population of cells regarding the functionality of the cells. Each subtype of CD4+ T cells has unique

properties and their individual role in atherosclerosis development is not fully elucidated. However, aiming at reduce the proinflammatory CD4+ T cells and/or increase the antiatherogenic CD4+ T cells still seems as a promising approach in for development of novel therapies in prevention of atherosclerosis. By increasing the knowledge of how specific CD4+ T cells subsets are activated by MHC class II and DCs it may be possible to more precisely activate the T cell population of interest.

Svensk sammanfattning

Hjärt- kärlsjukdomar är den vanligaste dödsorsaken i Sverige. Den bakomliggande orsaken till de allra flesta fall av hjärt- kärlsjukdom är vad som i vardagligt tal kallas åderförkalkning. Den medicinska termen för åderförkalkning är *ateroskleros*. Ateroskleros är en inflammatorisk sjukdom i artärerna som orsakas av att LDL-partiklar, det så kallade onda kolesterolet, ansamlas i kärlväggen, blir oxiderade och startar en inflammatorisk process. Inlagringen av LDL-partiklar och inflammatoriska celler gör att så kallade aterosklerotiska plack bildas vilka orsakar förträngningar i blodkärlen. De aterosklerotiska förträngningarna kan spricka vilket leder till att en blodpropp kan bildas inuti kärlen. Denna blodpropp orsakar ett stopp i blodets flöde och den vävnad som normalt försörjs av det tilltäppta kärlet får syrebrist. Om syrebristen inträffar i hjärtats kranskärl kallas det hjärtinfarkt, inträffar det i hjärnan kallas det stroke. Behovet av nya effektivare behandlingar av ateroskleros är stort. De behandlingar som finns i dagsläget går ut på att antingen sänka blodfetterna för att minska bildandet av nya plack eller att med kirurgi ta bort placken från kärlen.

Den inflammatoriska processen i kärlen involverar en mängd olika celler som tillhör kroppens immunförsvar. Immunförsvaret har som uppgift att skydda kroppen mot till exempel bakterie- och virus infektioner. Ibland händer det dock att immuncellerna aktiveras av ett kroppseget ämne vilket leder till autoimmunitet. Ateroskleros brukar ofta klassas som en autioimmun sjukdom eftersom inflammationen till stor dela är riktad mot oxiderat LDL. Immunförsvaret kan delas upp i två olika delar; det *medfödda* och det *specifika immunförsvaret*. Det medfödda immunförsvaret är kroppens första försvarslinje och det kan snabbt aktiveras då ett främmande ämne upptäcks i kroppen. Många av de celler som tillhör det medfödda immunförsvaret är förprogrammerade att ”äta upp” främmande ämnen och oskadlig göra dem. Det medfödda immunförsvaret är också delaktigt i aktiveringen av cellerna från det specifika försvaret, de så kallade T och B cellerna. Aterosklerostiska plack innehåller ett stort antal celler som tillhör det medfödda immunförsvaret men även en del T och B celler.

För ca 15 år sedan försökte forskare framkalla ateroskleros i kaniner genom att vaccinera dessa med oxiderat LDL och ett *adjuvans* (adjuvans är ett ämne som

används vid vaccinering för att få ett kraftfullare immunsvar). I motsats till vad man förväntade sig så fick de vaccinerade djuren mindre ateroskleros i sina kärl. Dessa resultat väckte tankar om att utveckla ett vaccin mot ateroskleros för att förebygga sjukdomen hos människor. På avdelningen för Experimentell Kardiovaskulär Forskning vid Lunds Universitet har man i drygt 10 år arbetet med att försöka identifiera den specifika del av LDL som framkallar ett skydd vid vaccinering. LDL är en komplex partikel som innehåller ett stort protein, *apoB-100*. Flera olika delar av apoB-100 har visat sig kunna minska utvecklingen av ateroskleros med upp till 60% när de används tillsammans med adjuvanset Alum vid immunisering av aterosklerosbenägna musmodeller. Syftet med *delarbete II* i den här avhandlingen var att försöka förstå vad det är som händer i immunförsvaret när möss vaccineras med det apoB-100 relaterade vaccinet *aBp210*. Eftersom aBp210 med största sannolikhet för första gången ska testas på människa under 2011, är det viktigt att förstår så mycket som möjligt om hur vaccinet fungerar, dels för att kunna göra det så effektivt som möjligt och dels för att veta vad man ska undersöka när man vill se om vaccineringen har fungerat. Resultaten i delarbete II visar att vaccinering med aBp210 aktiverar så kallade *regulatoriska T celler*. Regulatoriska T celler är en specifik sorts T celler som till skillnad från konventionella T celler dämpar inflammatoriska processer.

Tidigare forskning har visat att när man vaccinerar möss som har defekt lipidmetabolism med enbart adjuvantet Alum kan detta resultera i minskad utveckling av ateroskleros. I *delarbete I* har vi undersökt vad det är som gör att Alum kan ha en skyddande effekt. Alum-vaccinering leder, precis som vaccinering med aBp210, till att andelen skyddande regulatoriska T celler ökar. Resultaten visar också att Alum tar upp LDL som finns vid injektionsstället och att LDL då kan härma det apoB-100 relaterade vaccinet aBp210. Denna effekt ses dock endast hos möss som har defekt kolesterolomsättning eftersom LDL inte finns i vävnaderna vid normala lipidnivåer. Om det är så att Alum kan ha motsvarande effekt hos människor med kraftigt förhöjda LDL-nivåer i blodet kvarstår att undersöka.

För att kunna identifiera de personer som har en förhöjd risk att få hjärtinfarkt eller stroke är det viktigt att utveckla nya metoder för detta. I *delarbete VI* har vi undersökt om mängden regulatoriska T celler i blodet är kopplat till risken att utveckla hjärtinfarkt eller stroke. För att undersöka detta använde vi oss av frysta blodceller från Malmö Kost och Cancer studiens kardiovaskulära del. Vi valde ut 700 individer och jämförde mängden regulatoriska T celler och fann att de som senare insjuknade i hjärtinfarkt eller stroke hade färre regulatoriska T celler i blodet när de rekryterades till studien.

För att studera betydelsen av olika delar av immunförsvaret vid utvecklingen av ateroskleros kan man använda möss som dels har defekt kolesterolomsättning och dels har olika defekter i sitt immunförsvar. I *delarbete III, IV och V* har vi studerat tre olika proteiner som finns på cellerna som tillhör det medfödda immunförsvaret. I delarbete III har vi studerat betydelsen av FcγRIIB i utvecklingen av ateroskleros. FcγRIIB är en så kallad receptor som antikroppar kan binda till. När FcγRIIB aktiveras minskar aktiviteten i den cellen som receptorn sitter på. Om FcγRIIB saknas utvecklar mössen mer ateroskleros. Dessa resultat tyder på FcγRIIB skulle kunna vara ett möjligt mål för framtida läkemedel som syftar på att minska risken för hjärt- kärlsjukdomar.

I *delarbete IV* studerade vi vilken betydelse den antigenpresenterande molekylen *MHC klass II* har vid utvecklingen av ateroskleros. *Antigen* är ett annat namn för det främmande ämne som aktiverar cellerna i immunförsvaret. När ett antigen tagits upp av en immuncell bryts det ner i mindre delar. Dessa små delar kommer sedan bilda ett komplex tillsammans med MHC klass II och därefter visas upp på ytan av cellen, dvs antigenpresentation. Antigenpresentation är nödvändigt för att kunna aktivera vissa av T cellerna från det specifika immunförsvaret. I delarbete IV visade vi att avsaknad av MHC klass II gör att möss får mer ateroskleros. I *delarbete V* studerade vi den antigenpresenterande molekylen CD1d som också finns på immunceller. Vi visade att avsaknad av antigenpresentation via CD1d leder till en minskad förträngning i kärlen.

Sammanfattningsvis pekar resultaten i den här avhandlingen på att regulatoriska T celler har betydelse vid vaccinering för att hindra utvecklingen av ateroskleros. Vidare så kan kanske regulatoriska T celler komma att användas som en markör för att avgöra risken att utveckla hjärtinfarkt eller stroke. Dessutom visar resultaten att immunförsvaret kan vara ett lämpligt mål för framtida behandlingar för att minska utvecklingen av ateroskleros.

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