Regulatory Immune Responses and Repair Mechanisms in Atherosclerosis

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Abstract

Atherosclerosis is a chronic inflammatory disease characterized by the formation of lipid rich plaques in the arterial wall. Rupture of a plaque results in clinical manifestations such myocardial infarction or stroke. Atherosclerosis is a complex disease where both autoimmune responses towards atherosclerosis-related antigens and smooth muscle repair responses play important roles. This thesis contains studies focusing on both regulatory immune responses and tissue repair mechanisms in experimental models of atherosclerosis as well as in patient cohorts.

The first part of this thesis investigates the role of regulatory immune responses targeting plaque-related antigens. In paper I, we developed a matrigel-based method to characterize T helper 2 immune responses against human apolipoprotein B100 (ApoB100). We report that matrigel loaded with the antigen of interest can be used to measure antigen-specific immune cell accumulation and cytokine production. In paper II, we report that B cells pulsed with peptide 210 (p210) from ApoB100 coupled to the cholera toxin B subunit (p210-CTB) acquire a regulatory phenotype and induce Tregs *in vivo*. In the third paper, we unexpectedly found increased frequencies of circulating regulatory T cells in patients with prevalent cardiovascular disease. Our results indicate that the general immune cell activation in patients with prevalent cardiovascular disease can cause a compensatory increase in regulatory T cells to counteract the immune response.

In the second part of this thesis, we focused on the role of smooth muscle cells in atherosclerosis. In paper IV, we show that IL-22 is involved in controlling smooth muscle cell phenotype. More specifically, IL-22 deficient atherosclerotic mice develop smaller plaques with increased expression of contractile proteins. Increased numbers of smooth muscle cells remaining in a contractile phenotype in the media and decreased collagen content in the plaques could possibly contribute to the smaller plaque size observed in IL-22 deficient mice. Finally, in paper V, we present data suggesting that high levels of smooth muscle cell growth factors (platelet-derived growth factor, epidermal growth factor, heparin-binding epidermal growth factor) measured in plasma can reflect a fibrous plaque phenotype. In particular, high plasma levels of heparin-binding epidermal growth factor at baseline was associated with a decreased risk for developing a coronary event.

Key words: Atherosclerosis, Cardiovascular disease, T helper 2 cells, ApoB100, Regulatory B cells, Regulatory T cells, Smooth muscle cells, IL-22.

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Preface

Atherosclerosis is a chronic inflammatory disease where both the immune system and tissue repair mechanisms play important roles. Pro-inflammatory immune responses have been shown to promote atherosclerosis development but there is also evidence for a regulatory arm that dampens the inflammation and stabilizes the atherosclerotic plaque. During plaque stabilization, smooth muscle cells and the production of extracellular matrix proteins are pivotal.

In this thesis, I will present and discuss results concerning both regulatory immune responses and repair mechanisms, in the context of atherosclerosis. The first three studies are focused on regulatory immunity, in particular T helper 2 responses against atherosclerosis-related antigens and regulatory B and T cells. The fourth study links the two parts together and shows that the immune system can regulate tissue repair. Lastly, in the fifth study we investigate the role of smooth muscle cell growth factors as predictors for cardiovascular disease in man.

If I am able to make you fascinated about the intriguing world of immunity or interested in the mechanisms behind the disease that is the leading cause of death in our society, I have succeeded. However, I am also happy if you, except for the acknowledgement section, read the “populärvetenskaplig sammanfattning”.

Original Papers

The thesis is based on the following papers and manuscript and referred to in the text by their roman numerals indicated below:

I. Engelbertsen D, **Rattik S**, Knutsson A, Björkbacka H, Bengtsson E, Nilsson J. Induction of T helper 2 responses against human apolipoprotein B100 does not affect atherosclerosis in ApoE-/- mice. *Cardiovascular Research 103, 303-312, 2014*

II. **Rattik S**, Wigren M, Mantani PT, Söderberg I, Sundius L, Björkbacka B, Holmgren J, Fredrikson GN, Nilsson J. B cells treated with p210-CTB acquire a regulatory phenotype and induce regulatory T cells in vitro. *Manuscript*

III. **Rattik S**, Engelbertsen D, Wigren M, Mantani PT, Ljungcrantz I, Östling G, Persson M, Fredrikson GN, Björkbacka H, Nilsson J. Increased levels of regulatory T cells in patients with cardiovascular disease. *Manuscript* (*submitted)*

IV. **Rattik S**, Hultman K, Rauch U, Söderberg I, Sundius L, Ljungcrantz I, Hultgårdh-Nilsson A, Björkbacka H, Fredrikson GN, Nilsson J. IL-22 affects smooth muscle cell phenotype and plaque formation in apolipoprotein E knockout mice. *Manuscript (submitted)*

V. **Rattik S**, Wigren M, Björkbacka B, Fredrikson GN, Hedblad B, Siegbahn A, Bengtsson E, Schiopu A, Edsfeldt A, Dunér P, Grufman H, Gonçalves I, Nilsson J. High plasma levels of heparin-binding EGF is associated with a more stable plaque phenotype and reduced incidence of coronary events. *Arterioscler Thromb Vasc Biol. 2014 Oct 30. [Epub ahead of print]*

Published or Submitted Papers not included in the thesis

Saxena A, Björkbacka H, Ström Å, **Rattik S**, Berg KE, Gomez MF, Fredrikson GN, Nilsson J, Hultgårdh-Nilsson A. Mobilization of regulatory T cells in response to carotid injury does not influence subsequent neointima formation. *PLoS One. 7, e515565, 2012.*

Wigren M, **Rattik S**, Grönberg C, Söderberg I, Alm R, Sundius L, Ljungrantz I, Björkbacka H, Fredrikson GN, Nilsson J. Lack of ability to present antigens on MHC class II molecules aggravates atherosclerosis in ApoE-/- mice. *Submitted*

Wigren M, **Rattik S**, Hultman K, Björkbacka H, Fredrikson GN, Gonçalves, Bengtsson E, Hedblad B, Siegbahn A, Nilsson J. Decreased levels of stem cell factor in subjects with incident coronary events. *Submitted*

Abbreviations

AHR	Aryl hydrocarbon receptor
APC	Antigen presenting cell
ApoB	Apolipoprotein B
ApoE	Apolipoprotein E
ASC	Acute coronary syndrome
Au	Arbitrary units
BCR	B cell receptor
Breg	Regulatory B cell
CANTOS	Canakinumab anti-inflammatory thrombosis outcomes study
CCL	Chemokine (C-C motif) ligand
CD	Cluster of differentiation
CE	Cardiovascular event
CM	Chylomicrons
CPIP	Carotid plaque imaging project
CRP	C-reactive protein
CTB	Cholera toxin B subunit
CTLA4	Cytotoxic T lymphocyte antigen 4
CVD	Cardiovascular disease
CXCL	Chemokine (C-X-C motif) ligand
DAMP	Danger associated molecular pattern
DCs	Dendritic cells
EAE	Experimental autoimmune encephalomyelitis
ECM	Extracellular matrix

ECs	Endothelial cells
EGF	Epidermal growth factor
FoxP3	Forkhead box P3
GATA3	GATA-binding protein 3
H-Y	Y-chromosome-encoded minor histocompatibility antigens
HB-EGF	Heparin binding epidermal growth factor
HDL	High-density lipoproteins
HFD	High fat diet
HLA	Human leukocyte antigen
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HSP	Heat-shock protein
i.p.	Intraperitoneal
i.v.	Intravenous
IDL	Intermediate low-density lipoprotein
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL-10RA2	IL-10 receptor alpha 2
IL-22BP	IL-22 binding protein
IL-22R	IL-22 receptor
IL-22RA1	IL-22 receptor alpha 1
IL-22RA2	IL-22 receptor alpha 2
ILC	Innate lymphoid cells
IMT	Intima-media thickness
iNKT	Invariant natural killer T cell
iTregs	Induced regulatory T cells
L	Ligand
LAP	Latency-associated peptide
LDL	Low-density lipoproteins

LDLr	LDL receptor
LOX-1	Lectin-like oxidized LDL receptor-1
LTi	Lymphoid tissue inducer cells
MCP-1	Monocyte chemoattractant protein-1
MDA	Malondialdehyde
MDC	Malmö diet and cancer
MHC	Major histocompatibility complex
MI	Myocardial infarction
MMP	Matrix metalloproteinases
MOG	Myelin oligodendrocyte glycoprotein
MZ	Marginal zone
NK	Natural killer
NKT	Natural killer T cells
nTregs	Natural regulatory T cells
OT-II	Ovalbumin-transgenic
OVA	Ovalbumin
oxLDL	Oxidized LDL
PAMP	Pattern associated molecular pattern
PD	Programmed death
PDGF	Platelet derived growth factor
PEA	Proximity extension assay
PRR	Pattern-recognition receptors
RORγt	Retinoid-acid receptor-related orphan receptor gamma t
SMCs	Smooth muscle cells
SOCS3	Suppressor of cytokine signaling 3
SR	Scavenger receptor
STAT3	Signal transducer and activator of transcription 3
STEMI	ST-elevated myocardial infarction

SUMMIT	Surrogate markers for micro- and macrovascular hard endpoints for innovative diabetes tools
T-bet	T-box transcription factor
T2D	Type 2 diabetes
TCR	T cell receptor
TG	Triglycerides
TGF	Transforming growth factor
Th	T helper
TIF	T cell inducible factor
TLR	Toll-like receptors
TNF	Tumor necrosis factor
TRAF	TNF receptor-associated factor
Treg	Regulatory T cell
VCAM	Vascular cell adhesion protein
VLDL	Very low-density lipoproteins

Background

The pathology of atherosclerosis

Atherosclerosis, thickening of the arterial wall, is the underlying cause of most cardiovascular disease (CVD)-related mortalities. CVD includes myocardial infarction (MI) and stroke and taken together, CVD is the leading cause of death in western societies.

Ischemic stroke is mainly caused by either a ruptured intracerebral plaque or from an emboli originating from a carotid or cardiac plaque while rupture of a plaque in the coronary artery is the primary cause of an acute MI or unstable angina[1-3]. At such an event, the artery is rapidly occluded causing ischemia and loss of function of the heart or the brain.

The incidence of CVD continues to increase, predominantly because of the growing frequency of life-style related diseases such as diabetes and obesity. Together with hypertension, dyslipidemia and smoking, diabetes and obesity are major risk factors for developing CVD[4].

In this part, I will give a brief introduction to the biological processes resulting in the formation of an atherosclerotic plaque.

The healthy vessel

The vascular wall consists of three layers, tunica adventitia, tunica media and the inner layer, the intima (Figure 1). The outer adventitia contains fibroblasts, mast cells as well as different types of resident progenitor cells with the ability to differentiate into smooth muscle cells (SMCs) or endothelial cells (EC)[5,6]. The *vasa vasorum* supplies the adventitial layer with nutrients and is responsible for the exchange of soluble components[5]. The tunica media is build up by SMCs embedded in extracellular matrix (ECM) containing collagen type I, III, V and XVIII, fibronectin, proteoglycans and elastic laminae[7,8]. The ECM provides tensile strength and the vascular tone is controlled through the contractile abilities of SMCs[7]. Closest to the lumen, the intima layer consists of ECs surrounded by a basement membrane and ECM. The intima functions as a barrier but ECs are also important in regulating the vascular tone by the release of vasoactive molecules

such as nitric oxide (vasodilation) and endothelin (vasoconstriction)[9]. The crosstalk between cells in the different layers of the vessel wall is essential during both normal and pathological situations.

Lipoprotein metabolism

Elevated levels of plasma lipids are a well-known risk factor for developing atherosclerosis, even when other risk factors are absent. This is evident when studying patients with familial hypercholesterolemia[10]. Cholesterol and triglycerides (TG) are important plasma lipids with abundant biological roles. Due to its hydrophobic nature, these lipids need to be transported in the circulation as part of macromolecular lipoproteins.

Chylomicrons (CM) and very low-density lipoproteins (VLDL) are the main carriers of TG. In the intestine, TGs are packed together with apoplipoprotein B48 (ApoB48) to form CM that subsequently is released into the circulation. In the peripheral tissue, the fat component of CM is hydrolyzed by lipoprotein lipases to generate free fatty acids that are taken up by the tissue. The remaining structure is called CM remnants that in turn are taken up by the liver via the low-density lipoproteins (LDL) receptor (LDLr).[11] VLDL is formed in the liver by the combination of TG, cholesterol and apolipoprotein B100 (ApoB100). However, in mice, VLDL also contains the truncated version ApoB48. VLDL is subjected to lipolysis, creating first intermediate low-density lipoproteins (IDL) and subsequently LDL. Because of TG lipolysis, LDL is enriched in cholesterol. LDL is considered "the bad cholesterol" and a high level of LDL is a well-known risk factor for CVD.[11]

On the other hand, high-density lipoproteins (HDL) is recognized as "the good cholesterol" and plasma levels of HDL are reduced in patients with CVD[12,13]. HDL mediates reverse cholesterol metabolism, transporting cholesterol to the liver for catabolism and secretion through the bile.

Plaque development and rupture - an overview

The response to retention hypothesis

It was recognized early on that atherosclerotic plaques contain a high amount of cholesterol[14]. In particular, LDL particles have been shown to be retained in the arterial wall by the interaction between electropositive parts of ApoB100 and negatively charged ECM proteins, especially biglycan and decorin[15]. This is part of the response-to-retention hypothesis, proposed in 1995 by Tabas and Williams to be the key initiating event for the formation of an atherosclerotic plaque[16].

While held in the arterial wall, the LDL particles become modified by metal ions and reactive oxygen species as well as via enzymatic reactions by myeloperoxidases and lipoxygenases[17]. Modification of the phospholipids forms oxidized LDL (oxLDL) as well as pro-inflammatory mediators such as lysophosphatidylcholine. Oxidation of LDL also generates reactive aldehyde adducts, such as malondialdehyde (MDA), on ApoB100 creating a modified-self protein. Immune responses targeting modified ApoB100 derived peptides will be discussed in a later section.

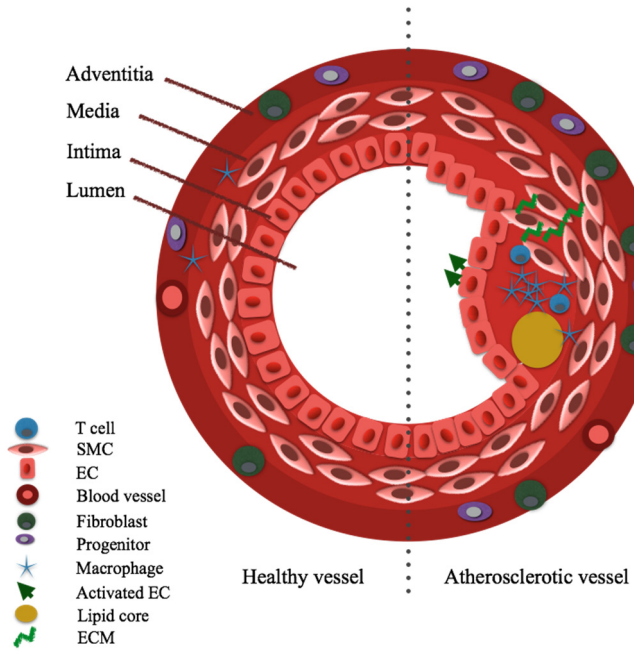

Figure 1. A simplified overview of the healthy vessel in comparison to an atherosclerotic vessel.

In brief, plaque development is initiated by oxLDL and oxLDL derived factors generated in the oxidation process, which activate ECs and macrophages via binding to several pattern-recognition receptors (PRRs) such as scavenger receptors and toll-like receptors (TLRs)[18]. This results in the production of pro-inflammatory cytokines leading to the expression of adhesion molecules on ECs and the subsequent recruitment of neutrophils and monocytes from the circulation. Monocytes that enter the tissue turn into macrophages and together with resident macrophages these cells will engulf oxLDL as an attempt to clean the tissue from the insulting agent. During this inflammatory process, SMCs will become activated to initiate a tissue repair mechanisms aiming to restore the normal vascular function and structure. The SMCs will dedifferentiate and start to migrate into the intima to produce ECM that subsequently can build up a protective fibrous

cap. As LDL accumulation lasts, macrophages will continue to take up LDL and consequently turn into lipid-loaded foam cells. Foam cells easily become necrotic and because of inefficient clearance of dying cells during atherogenesis, a necrotic core will form in the growing plaque.[6,19]

Plaque rupture

Atherosclerotic plaques develop during decades and the presence of a "stable plaque" *i.e.* a plaque containing high levels of SMCs and a thick fibrous cap may go unnoticed for an entire life span (Figure 2). An opposite mechanism causing plaque instability is activated macrophages producing several types of matrix metalloproteinases (MMPs) that degrade ECM proteins. If the fibrous cap is degraded and the plaque ruptures, tissue proteins will get exposed and initiate a thrombus formation. The thrombus can interrupt the blood flow locally or embolize and travel in the circulation until it gets trapped in smaller arteries. At such an event, the artery is rapidly occluded causing ischemia and loss of function of the heart or brain. The balance between SMCs and ECM production and inflammation-controlled ECM degradation will determine if the plaque stays silent and stable or turn into an "unstable" plaque phenotype with a high risk of causing symptoms (Figure 2).

Next, I will go into more detail regarding some of the mechanisms that are of importance during atherosclerosis development.

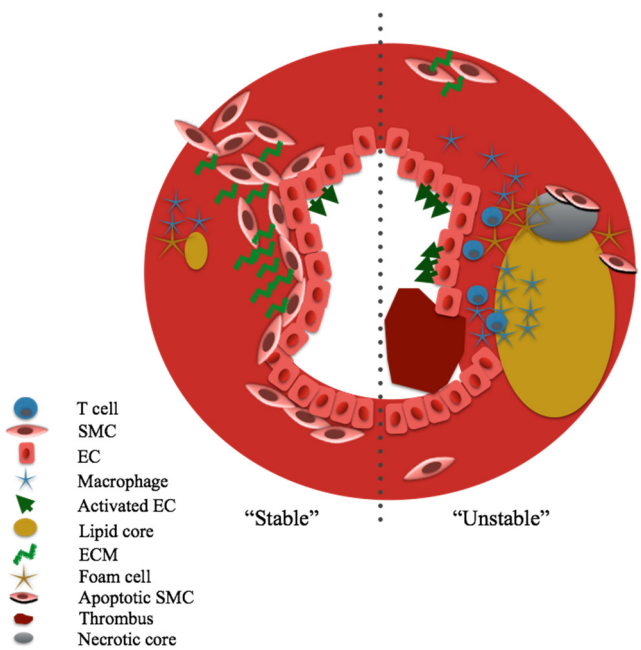

Figure 2. Characteristics of the "stable" and "unstable" plaque phenotype

Innate immunity - the first steps of atherogenesis

Innate immunity is the first line of defense against invading pathogens. Compared to the slower adaptive part of the immune system (discussed later on), cells of the innate immunity recognize broader pathogen associated molecular patterns (PAMPs) or danger associated molecular patterns (DAMPs) instead of specific peptides. The initiation of an inflammatory response is mediated through the recognition of PAMPs and DAMPs via PRRs expressed on cells such as macrophages and endothelial cells.

Modified LDL shares some PAMP/DAMP epitopes and can be recognized by a wide range of PPRs[20]. In particular, oxLDL have been suggested to be part of a subclass of DAMPs including oxidation-specific epitopes[20]. Binder *et al* showed that Pneumococcal vaccination could inhibit atherosclerosis development suggesting mimicry between PAMP epitopes in *Streptococcus Pneumonia* and oxLDL[21].

Endothelial cells express, among others, TLR1,2 and 4 which have been shown to bind oxLDL and components thereof[18]. Activation of endothelial cells via oxLDL and TLR signaling results in the expression of E-selectin and vascular cell adhesion molecule 1 (VCAM-1) and the subsequent release of chemokines (*e.g.* CCL2, CCL5 and CXCL10) to recruit T cells and monocytes from the bloodstream[18]. Endothelial cells also secrete macrophage colony-stimulating factor that induce monocyte differentiation into macrophages and the upregulation of scavenger receptors (SR).

Macrophages

Macrophages are a heterogeneous cell population with important functions in innate immunity and during atherosclerosis development. The main function of macrophages and its scavenger receptors is to clear cell debris as well as microbes from the surrounding tissue. Some scavenger receptors *e.g.* CD36, lectin-like oxLDL receptor 1 (LOX-1) and SR-A can also directly bind oxLDL[18,22]. Macrophage uptake of oxLDL stimulates the production of pro-inflammatory mediators and the formation of lipid-loaded cells that when overloaded and chronically stimulated turn into foam cells[18,23]. Intracellular cholesterol can also activate the inflammasome and the production of interleukin(IL)-1β[24]. In the early stage, most of the plaque macrophages originate from recruited monocytes while in more established atherosclerosis, local proliferation has been reported to regulate the accumulation of macrophages[25].

Macrophages are very plastic meaning that cytokines and microbial products in the local milieu affect their activation status. T helper 1 (Th1) cell cytokines (mainly interferon γ (IFNγ)) are thought to induce a “classical” activation profile called M1 macrophages while Th2 cytokines (IL-4, IL-13) induce “alternatively”

activated macrophages (M2). M1 macrophages are potent effector cells, have a high rate of phagocytosis and produce mainly pro-inflammatory cytokines such as tumor necrosis factor α (TNFα), IL-6 and IL-12 whereas M2 macrophages dampens Th1 inflammation and promote vascular repair by the production of IL-10 and transforming growth factor β (TGFβ)[26].

Vascular repair mechanisms

SMC migration and proliferation are normal and essential processes during embryonic development and in the response to injury. As will be discussed in this section, SMC migration and production of ECM proteins are pivotal for the stabilization of the atherosclerotic plaque. On the other hand, extensive SMC repair or the failure to switch back to a contractile phenotype results in pathogenic vascular remodeling in for example restenosis.[27] This means that the switch between a contractile and synthetic SMC phenotype needs to be carefully regulated.

SMC phenotypic switch

In the tunica media of adult animals, vascular SMC exists as highly differentiated and specialized cells with the main function to regulate blood pressure. At this stage, the cells have a spindle-shaped phenotype and a number of genes such as α-actin[28], smoothelin[29] and SM myosin heavy chain[30,31] have been identified to be preferentially expressed in the differentiated SMC.[32] For a list of suggested SMC phenotypic genes or markers, see Table I.

Even though contractile SMCs can be considered differentiated, it is still a highly plastic cell population[32]. The production of cytokines and growth factors by oxLDL-activated EC and macrophages will stimulate dedifferentiation of medial SMCs into a synthetic phenotype, acquiring an epithelioid shape. This is known as the SMC phenotypic switch which is associated with decreased expression of contractile SMC genes as well as increased cell proliferation and production of growth factors. Dedifferentiated SMCs begin to migrate from the media into the intima and start to synthesize a broad repertoire of ECM proteins such as elastin and different types of collagens, building up a protective fibrous cap.[33] Interestingly, oxLDL have also been shown to directly suppress the expression of α-actin and SM myosin heavy chain (SM-MHC) in SMCs[34].

It is difficult to pinpoint genes that are exclusively expressed in synthetic SMCs and hence, down-regulation of contractile genes is suggested to be a characteristic of the dedifferentiated synthetic SMC phenotype. However, a few genes or markers that are preferentially expressed in synthetic SMCs have been suggested (see Table I for a summary).

Table I: Suggested SMC phenotypic markers			
Gene	**Function**	**SMC phenotype**	**Selected reference(s)**
α-actin	Contraction	Contractile	[28]
SM myosin heavy chain	Contraction	Contractile	[30,31]
Smoothelin	Regulate contraction	Contractile	[29,35]
h-Caldesmon	Regulate contraction	Contractile	[36]
Meta-Vinculin	Regulate contraction	Contractile	[37]
Calponin	Regulate contraction	Contractile	[38]
SM22α	Regulate contraction	Contractile	[39,40]
l-Caldesmon	Regulate contraction	Synthetic	[41]
Vimentin	Type III intermediate filament; maintains cell shape and integrity of cytoplasm	Synthetic	[42-44]
Nonmuscle myosin heavy chain B	Actin–myosin force generation	Synthetic	[45]
Cellular-retinol binding-protein-1	Involved in retinoid metabolism	Synthetic	[46,47]
Tropomyosin 4	Actin-binding protein, regulates actin-mechanics	Increased in Synthetic	[48,49]
MMP isoforms	Degrading ECM, migration	Synthetic	[50]
PDGF	Migration	Synthetic	[50]
Genes associated with ECM production	ECM production	Synthetic	[50]

Table adapted and modified from references [38,50].

SMC growth factors

Several soluble factors present in the atherosclerotic plaque are involved in determining the phenotype of SMCs. In particular, platelet-derived growth factor (PDGF), secreted by both EC and activated macrophages, have been shown to down-regulate α-actin expression and induce SMC proliferation and migration[51]. Studies investigating the role of PDGF in the context of neointima formation have reported reduced neointima size and decreased SMC migration when neutralizing PDGF[52] while infusion of PDGF increased intimal thickening after arterial injury in rats[53]. In atherosclerosis, a recent study demonstrated a delayed formation of the fibrous cap in the absence of PDGF[54]. These studies support a plaque-stabilizing role for PDGF but also suggest that other SMC growth factors play a role in fibrous cap formation. Additionally, mediators such as TNFα, epidermal growth factor (EGF) and heparin-binding EGF (HB-EGF) have been shown to be involved in vascular SMC migration and proliferation. HB-EGF has been reported to be more potent than EGF and TNF-α in stimulating SMC proliferation and migration

due to its interaction with cell surface heparin sulphate[55]. TNFα has been shown to have different effects depending on the SMC phenotype. TNFα treatment induces proliferation in spindle-shaped SMCs while inducing apoptosis in more epithelioid SMCs[56].

In contrast to the SMC growth factors, TGFβ isoforms promote a contractile SMC phenotype. This is illustrated by studies showing that TGFβ1 and TGFβ2 increase α-actin, SM-MHC and SM-calponin expression levels[57-59]. However, the effect of TGFβ on SMCs seems to dependent on the type of TGFβ receptor that is expressed. SMCs isolated from healthy vessel express the type I and II TGFβ receptor while SMCs in the plaque have a reduced type II receptor expression and instead preferentially express the TGFβ type I receptor. Interestingly, signaling via the type I receptor results in increased ECM production, which is not observed in type II TGFβ receptor dominated cells[60,61].

ECM degradation and plaque vulnerability

To be able to migrate from the intima to the media, SMCs produce certain MMPs. MMPs are a family of 23 related enzymes sharing the dependence of Zn^{2+} for their catalytic function[62]. Their function is to degrade ECM proteins, regulate cell migration as well as to modify and activate soluble or surface proteins including cytokines and growth factors[62]. This means that MMPs contribute both to plaque vulnerability, by the degradation of ECM, and to tissue repair, by stimulating SMC migration. PDGF stimulates the production of MMP-2 in SMCs which enable the migration of medial SMCs into the intima[63,64]. Moreover, MMP-2 has been associated with a more stable plaque phenotype while a high expression of MMP-1, MMP-8 and MMP-9 are associated with more vulnerable plaques[65,66]. On the other hand, atherosclerotic apolipoprotein E (ApoE) deficient mice lacking MMP9 or MMP2 develop decreased amount of atherosclerosis[67,68]. Furthermore, MMP3, as well as other enzymes, has been implicated in cleaving pro-HB-EGF to release the mature form of HB-EGF and in that manner MMP3 contributes to SMC migration[69].

Interestingly, different macrophage subtypes have been shown to secrete a diverse repertoire of MMPs. For example, M1 macrophages have increased gene expression MMP1, MMP2, MMP3, MMP10 and MMP14 compared to M2 macrophages. This may explain the increased collagenolytic activity (specifically due to increased MMP1 levels) of M1 macrophages and result in different availability of cytokines and growth factors[70].

Taken together, this emphasizes the important balance between ECM production by SMC and the ECM degrading pro-inflammatory responses in determining the vulnerability of the atherosclerotic plaque.

Type 2 diabetes

Lifestyle and genetic factors influence glucose homeostasis and can cause hyperglycemia. This is known as insulin-resistance, a key characteristic for type 2 diabetes (T2D). At first, this is compensated by an increased production of insulin from the pancreatic beta cells but after a prolonged period of time, the beta cells will no longer compensate for the increased requirement of insulin, resulting in hyperglycemia and developed T2D[71].

Patients with T2D suffer from both micro- and macrovascular complications and display a 2-4 fold increased risk for suffering from a cardiovascular event (CE)[72]. The underlying mechanisms remain to be fully understood but an atherogenic lipoprotein profile, chronic low grade inflammation, endothelial and platelet dysfunction as well as impaired fibrous repair have been proposed as potential explanations[73-76]. Accordingly, plaques from T2D subjects contain more inflammatory cells and it has become generally accepted that metabolically driven inflammation contributes to the increased incidence of CVD in T2D[75,77]. In paper III, I present a study investigating the involvement of T regulatory cell (Tregs) levels in CVD among T2D subjects.

Adaptive immune responses in atherosclerosis

The involvement of innate immune responses in atherosclerosis is well established. The growing atherosclerotic plaque contains lipids, macrophages, mast cells and migrating SMCs but also cells from the adaptive part of the immune system. Dendritic cells (DCs), T cells and B cells have been found in the developing intimal plaque and tertiary lymph node structures have been shown to form in the adventitia, close to advanced atherosclerotic plaques.[18] Although, tertiary lymphoid structures have not yet been observed in human plaques[78].

APC - the bridge between innate and adaptive immunity

The main function of antigen-presenting cells (APCs) is engulfing and processing protein antigens to be able to present them as short peptides on major histocompatibility complex (MHC) I or II to $CD8^+$ and $CD4^+$ T cells, respectively. DCs are the most important APC and the cells are present in various tissues. T cell activation mainly occurs in the draining lymph nodes and priming of a T cell requires three signals. First, the T cell receptor (TCR) of that particular T cell needs to recognize its specific peptide antigen presented on the MHC and second, the T cell must receive the correct co-stimulatory signals (e.g. CD28 on the T cell interacting with CD80 or CD86 on the APC). During homeostatic conditions,

antigens are presented by APC in a tolerogenic fashion that does not activate the corresponding T cell. In that setting, APCs express low levels of MHC and co-stimulatory molecules. On the other hand excessive innate immune activation generates danger signals (e.g. via DAMPs and TLR activation) that extensively activate DCs to upregulate co-stimulatory molecules and increase the presentation of peptides on MHCII. This will break the tolerance and result in activation of adaptive immunity. The third signal, i.e. cytokines produced by the APC, will determine what type of T cell response that is generated.[79] The outcome of different T cell responses in atherosclerosis is discussed in the upcoming section.

DCs can also directly promote tolerogenic responses. If activated in the presence of IL-10 or TGFβ, DCs acquire a tolerogenic phenotype characterized by its preferential ability of inducing Treg differentiation and the production of IL-10.[80] Tolerogenic responses can also be generated by the uptake of antigens from dying cells or by signaling via specific co-inhibitory molecules on T cells and DCs (e.g. cytotoxic T-lymphocyte-associated protein 4 (CTLA4) interacting with CD80/86 or Programmed-death (PD) 1-PD-Ligand(PD-L)1/2 interactions).

T cells in atherosclerosis

T cells develop in the thymus through a series of events known as positive and negative selection. Positive selection determines whether the T cell is going to express CD4 or CD8 and after the next step, negative selection, only T cells expressing a TCR that do not bind with high affinity to tissue-restricted self-antigens are allowed to enter the circulation. T cells that bear a TCR with high affinity for self-peptides are either deleted by apoptosis or turned into Forkhead box P3 (FoxP3)-expressing Tregs. This is known as central tolerance and if this process is not working properly the individual will suffer from severe autoimmunity.[81,82]

It was early on discovered that $CD4^+$ and $CD8^+$ T cells can be found in both human and mouse plaques, with a predominance of $CD4^+$ over $CD8^+$ T cells[83-85]. The important role for $CD4^+$ T cells were illustrated by depleting $CD4^+$ T cells in atherosclerotic mice, which results in reduced amount of disease while transfer of $CD4^+$ T cells into mice lacking T and B cells (mice with severe combined immunodeficiency) accelerated atherosclerosis development. Since then, the involvement of different T cell subtypes has been extensively studied and I will here briefly review the role of some important $CD4^+$ Th populations in atherosclerosis. $CD8^+$ T cells and natural killer (NK)-T cells are out of the scope of this thesis will therefore not be further discussed.

Th1

Th1 reactions are activated in response to intracellular pathogens. Production of IL-12 by the DC during the T cell priming phase results in differentiation towards the Th1 lineage. Th1 cells are characterized by T-box transcription factor (T-bet) expression and production of IFNγ and are thought to drive atherosclerosis development. This conclusion was first based on the fact that cells isolated from atherosclerotic plaques preferentially produce IFNγ[86]. The role of Th1 cells have also been extensively studied in several mouse models where blocking of Th1 effector cytokines and its signaling (IFNγ, IFNγ receptor, IL-12) as well as genetic deficiency in T-bet[87-91] results in decreased amount of disease while administration of effector cytokines has the opposite effect.

How can then Th1 responses promote atherosclerosis development? IFNγ activates macrophages to increase the production of nitric oxide, MMPs and pro-inflammatory cytokines. IFNγ also induces the expression of adhesion molecules and chemokines by ECs as well as inhibits collagen synthesis by SMCs[92]. Taken together, Th1 responses promote both plaque development and destabilization.

Th2

Th2 responses are provoked against extracellular pathogens or allergens. Lack of IL-12 or production of IL-4 will induce expression of the transcription factor GATA binding protein 3 (GATA3) and polarization towards a Th2 phenotype[80]. Th2 cells are characterized by the production of IL-4, IL-5 and IL-13 but reports regarding the role for Th2 cells in atherosclerosis have been inconsistent. One study performing bone marrow transfer of IL-4 deficient cells reported decreased amount of atherosclerosis while IL-4 deficiency either on the *Apoe*$^{-/-}$ or *LDLr*$^{-/-}$ background had no effect on disease development[93,94]. Bone marrow transfer using IL-5 deficient cells resulted in increased atherosclerosis associated with the reduction in natural antibodies targeting oxidation epitopes and IL-13 deficient mice also developed increased atherosclerosis possibly by acting on macrophages[95]. However, these findings are difficult to interpret because other cell populations such as innate lymphoid cells (ILCs) have been shown to produce high levels of IL-5, IL-4 and IL-13[96]. In patients, high levels of circulating IL-4 producing T cells are associated with a reduced risk of future CE[97].

Th17

IL-17 producing T cells (Th17 cells) were discovered when it was shown that mice lacking Th1 immunity still were susceptible to autoimmune disease[98]. Th17 was shown to be a specific lineage of cells that was dependent on IL-6 and TGFβ for its differentiation while IL-21, IL-23 and IL-1β also have been shown to be important for Th17 proliferation and maintenance of effector functions,[99-101]. Retinoid-acid receptor-related orphan receptor gamma t (RORγt) is the master

regulator transcription factor of Th17 differentiation and Th17 cells produce the signature cytokine IL-17 but also IL-22.

Studies of the role of IL-17 and Th17 cells in atherosclerosis have provided contradictory results[102]. An atherosclerotic environment and in particular oxLDL, have been shown to promote the production of IL-6 and result in a subsequent increase in IL-17 producing T cells[103]. Blocking IL-17, either via adenoviral delivery of a soluble IL-17 receptor[104], administration of a blocking antibody[105] or using mice deficient in the IL-17 receptor[106] resulted in reduced atherosclerosis. Moreover, IL-17 deficient mice exhibited similar extent of disease in one study while decreased amount of atherosclerosis was reported in another[107,108]. In contrast, Danzaki *et al* reported accelerated atherosclerosis in *IL-17*$^{-/-}$*Apoe*$^{-/-}$ mice after 8 weeks of high fat diet (HFD) and when administering IL-17, decreased atherosclerosis was observed[109,110]. Similarly, deletion of suppressor of cytokine signaling 3 (SOCS3) in T cells increased signal transducer and activator of transcription 3 (STAT3) signaling and IL-17 production which resulted in decreased atherosclerosis[110]. The regulatory role of IL-17 has been suggested to depend on its ability to decrease VCAM-1 expression and increase the production of collagen and hence, the formation of a fibrous cap[107,110-112].

The role of IL-17 and Th17 cells in atherosclerosis require further investigations. Interestingly, previous studies examining Th17 cells in atherosclerosis base their experiments on IL-17 while IL-22, which is also a major Th17 cytokine, has not (until now, in paper IV) been investigated in atherosclerosis.

Tregs

There is a reciprocal relationship between Th17 cells and Tregs. TGFβ is involved in Th17 polarization when IL-21 and IL-6 is present but TGFβ (in combination with IL-2) can also induce Treg differentiation[101]. Tregs are immunosuppressive cells that have been reported to be a protective cell population in atherosclerosis. Tregs are generally characterized as $CD25^{hi}FoxP3^{+}CD4^{+}$ T cells while human Tregs also express diminished levels of the IL-7 receptor, CD127[113-115]. Tregs can be either generated in the thymus *i.e.* natural or thymic Tregs (nTregs) or induced in the periphery, *i.e.* induced Tregs (iTregs). The transcription factor Helios has been suggested as a marker to distinguish nTregs from iTregs[116,117] but this has more recently been questioned[118,119].

Accumulating evidence suggests a protective role for Tregs in atherosclerosis and this have been extensively investigated in both experimental animal models as well as in clinical cohorts. In mice, expanding the number of Tregs via adoptive transfer or treatment with Treg-inducing anti-CD3 antibodies reduce atherosclerosis development[120,121] while removing Tregs by utilizing an anti-CD25 antibody or genetic knock out models exacerbate the disease[120,122]. Interestingly,

the latter study implicated Tregs in modulating lipoprotein metabolism by regulating the clearance of VLDL and CM from the circulation[122].

Clinical studies have reported conflicting results regarding the role of Tregs in CVD, possibly because of the difficulties in defining human Tregs. Most studies have reported decreased levels of Tregs ($CD25^{+}FoxP3^{+}$, $FoxP3^{+}$) in patients with CVD and high levels of circulating Tregs have been associated with a reduced risk for incident CVD[123-126]. In contrasts, Ammirati and colleagues reported increased levels of $CD25^{+}CD127^{low}$ Tregs in patients with ST-elevated MI (STEMI)[127]. Also, the suppressive function of Tregs isolated from both hypercholesterolemic mice and patients with CVD have been shown to be compromised[123,128]

How do Tregs protect against atherosclerosis? Tregs act by several immunosuppressive pathways to suppress both T effector cells and APCs. I will briefly mention some of the suppressive mechanisms by which Tregs regulate an immune response but it is important to note that all Treg subpopulations might not utilize all these pathways. For example, T regulatory cells type 1 cells are mainly dependent on IL-10 release while Th3 cells secrete TGFβ. Also, the particular circumstance may determine which mechanisms that are used.

1. T cell proliferation is dependent on IL-2. The high surface expression of CD25 (the receptor for IL-2) on Tregs allows the cells to consume IL-2 and thereby reduce the availability resulting in reduced T cell proliferation[129].
2. The production of immunosuppressive mediators such as IL-10, IL-35 and TGFβ suppress the activation of several effector cell types[130]. Except for being anti-inflammatory, *i.e.* inhibiting the maturation of APCs, proliferation of T effector cells and mast cell activation, IL-10 has also been shown to regulate cholesterol metabolism by reducing VLDL and LDL levels in plasma[131,132]. Moreover, Tregs have been shown to express the membrane-bound form of TGFβ and latency associated peptide (mTGFβ/LAP) that also has been implicated in the suppressive mechanism[133-135].
3. Another molecule expressed on Tregs is CTLA4. CTLA4 is involved in the immunosuppression by cell-cell mediated contact via the interaction of CTLA4 with CD80 and CD86 on the APC resulting in co-inhibition rather than co-stimulation[130,132].
4. Tregs can also directly kill effector cells and APC via granzyme B- and perforin-dependent mechanisms[136,137].

Via these (and other) mechanisms, Tregs regulate DC maturation, inhibit T effector cell activation and proliferation, induce M2 macrophages and impede foam-cell formation (by down regulation of scavenger receptors) as well as reduce

EC activation which together results in decreased plaque inflammation and enhanced plaque stability[138].

B cells in atherosclerosis

B cells are divided in B1 and B2 B cells where B1 cells are an innate B cell population while B2 cells are involved in both innate and adaptive responses.

B1 cells are resident mainly in pleural and peritoneal cavities, express TLRs and are the main producers of natural antibodies (Immunoglobulin (Ig) A, IgM). The B cell receptor (BCR) of B1 cells recognizes both microbial- and self-antigens[139]. For example, B1 cells have been shown to produce autoreactive natural IgM antibodies recognizing phosphorylcholine on apoptotic cells and oxLDL, but not native LDL[140]. B1 cells are characterized as $CD19^{+}B220^{lo/mid}IgM^{hi}IgD^{dull}CD43^{+}CD11b^{+}$ and then further subdivided into $CD5^{+}$ B1a and $CD5^{-}$ B1b cells[141].

B2 cells includes follicular B cells ($CD19^{+}B220^{+}IgM^{dull}IgD^{hi}CD21^{mid}CD23^{+}$) and marginal zone (MZ) B cells ($CD19^{+}B220^{+}IgM^{hi}IgD^{dull}CD1d^{hi}CD21^{hi}CD23^{-}$) that are primarily resident in lymphoid tissue[141]. Follicular B cells undergo isotype class switching and affinity maturation in response to T cell dependent antigens and can then turn either into plasma cells or memory B cells. Compared to follicular B cells which are mainly involved in adaptive immune responses, MZ B cells have been shown to be involved in innate immune responses[142]. MZ B cells reside in the spleen and can rapidly respond to soluble antigens in the circulation[141,142]. Because of their high expression of CD1d, MZ B cells are particularly important in presenting lipid antigens to invariant NKT cells[143].

IgM targeting oxLDL and IgM producing B1a cells have been shown to be protective in atherosclerosis. For example, splenectomized mice develop increased amount of atherosclerosis, which is abolished after transfer of B1a cells but not B2 cells.[141,144-146] B2 cells have been considered pro-atherogenic because anti-CD20 treatment that primarily depletes B2 cells, results in decreased atherosclerosis and transfer of B2 cells can increase disease development[147].

Regulatory B cells

Except for the production of antibodies, B cells have been ascribed additional functions including antigen presentation, cytokine production and immune modulation. It was discovered that a population similar to Tregs exists in the B cell compartment. This cell population is known as regulatory B cells (Bregs), characterized by the production of IL-10 and the capability of inducing and maintaining Tregs.[148] Research regarding Bregs is difficult because of the lack of a specific transcription factor that defines the lineage. Also, several surface markers have been proposed to describe Bregs suggesting that there might not be only one

Breg population. For example $CD1d^{hi}CD5^{+}$ Bregs produce IL-10 and suppress inflammation in a contact hypersensitivity model, $CD1d^{hi}CD21^{hi}CD23^{-}CD24^{hi}IgM^{hi}IgD^{lo}$ cells delay colitis development and $CD19^{+}CD1d^{hi}CD21^{hi}CD23^{hi}CD24^{hi}IgD^{hi}IgM^{hi}$ Bregs suppress the development of collagen-induced arthritis, antigen-induced arthritis and lupus[148-151]. Additionally, CD40-CD40L, TLR signaling and mTGFβ/LAP surface expression have been shown to be of importance for the suppressive actions of some Bregs[148,152,153]. Recently, the involvement of gut-microbiota driven production of IL-1β and IL-6 in the induction of functionally active Bregs was emphasized[154].

Modulating the immune system to treat atherosclerosis

The ultimate goal for treatment of autoimmune and chronic inflammatory diseases is to achieve persistent tolerance against the specific autoantigen, without overall compromising the immune system. Several immune modulating therapies have been suggested and tested in experimental atherosclerosis but none has yet reached the clinic.

Immunization induces a rapid and long-lasting pool of memory B and T cells that immediately can get activated to clear a pathogen if the host gets infected. One way to induce such a protection is to inject attenuated viral particles together with adjuvant. If the antigen in the vaccine is composed of a non-immunogenic molecule, the immunization can be performed with an adjuvant, for example something that activates TLR-signaling, to further potentiate the immune response. Adjuvants also have the capability of skewing the immune reaction to a Th1 or Th2 response.[155]

During recent years, several vaccine strategies have been developed to target not only infectious diseases but also autoimmunity and cancer. These diseases have the opposite requirements; in cancer, the goal is to potentiate an immune response against the tumor cells while in autoimmunity, it is to induce tolerance against the autoantigen to inhibit autoreactive T cells.

Antigen(s) in atherosclerosis

The complexity of atherosclerosis, compared to other autoimmune diseases, is the fact that there might not only be one antigen involved in the pathogenesis. Also, there is a considerable non-lymphocyte mediated part of the disease as mice deficient in both T and B cells still develop advanced lesions, although at a slower rate[156]. Several antigens have been suggested, both endogenous autoantigens and exogenous antigens derived from microbes, the latter mainly thought to be

involved by mimicry. Two autoantigens that have been shown to be of particular importance are heat-shock protein 60 (HSP60) and ApoB100.

HSP60

DAMPs are molecules that are normally hidden intracellular but released upon injury or stress. One such group of molecules is HSPs. Several HSPs such as HSP47, HSP70 and HSP60/65 have been implicated in atherosclerosis[157,158].

Early evidence for the involvement of HSPs in atherosclerosis came from a study reporting acceleration of atherosclerosis in rabbits after immunization with microbial HSP65[159]. Because of the high degree of homology of HSP65 between species it was suggested that this might be explained by a cross-reaction between microbial HSP65 and the mammalian HSP60. HSP60 is expressed upon injury on for example ECs and this is thought to initiate an inflammatory response and drive atherosclerosis development[160]. Moreover, serum levels of soluble HSP60, released from damaged cells, and antibodies against microbial HSP65 are elevated in subjects with atherosclerosis[161,162] and immunization of mice with HSP65 enhanced disease development[163,164].

ApoB100

Another important antigen in atherosclerosis is ApoB100, the major protein in LDL. In particular, both native and modified peptides of ApoB100 have been extensively studied as autoantigens in atherosclerosis. LDL oxidation is thought to give rise to modifications not only on the lipid part of LDL but also on ApoB100. For example MDA modified ApoB100 peptides are recognized by the immune system and therefore thought to be important as antigens in atherosclerosis.

Three decades ago, two groups showed that immunization of rabbits with modified and native LDL did not increased the amount of atherosclerosis but instead inhibited disease development[165,166]. Since then, oxLDL specific T cells have been isolated from human plaques and mouse and patients with CVD have antibodies against oxLDL that are associated with progression of the disease, further indicating that oxLDL is an important antigen in atherosclerosis[86,167,168]. Moreover, immunizing mice with oxLDL results in T cell clones that recognize LDL and ApoB100 and blocking T cells with that particular TCR inhibit atherosclerosis development[169]. To find which epitopes in LDL and oxLDL that are targets for the immune response, plasma from human subjects were screened for autoantibodies targeting peptides derived from ApoB100. It was shown that several peptide sequences from ApoB100, both native and MDA modified, were targeted by autoantibodies in human subjects[170]. In particular, peptide number 45 and 210 (p45 and p210, respectively) were targeted by autoantibodies and patients with CVD had lower levels of these antibodies indicating that immune responses against these epitopes can be beneficial[171,172]. Moreover, treating mice with an antibody against MDA modified p45 induced plaque regression and several immunization

studies using p210 as the antigen have reported inhibition of atherosclerosis development[173-175]. Antigens in atherosclerosis are further discussed in the "Results and Discussion" chapter.

Induction of tolerance

As mentioned earlier, T cells develop in the thymus through a series of events known as positive and negative selection. During negative selection, thymocytes expressing a TCR that binds to a self-peptide expressed on MHCII with high affinity go into apoptosis, "clonal deletion", or turn into Tregs, "clonal diversion"[82]. This is known as central tolerance. However, central tolerance cannot eliminate all self-reactive thymocytes and therefore another line of defense, known as peripheral tolerance, exists to limit T cell reactions towards autoantigens and non-pathogenic allergens. Peripheral tolerance consists of anergy (i.e. induction of unresponsive T cells), clonal deletion as well as the function of regulatory immune cell populations such as tolerogenic DCs, Tregs and Bregs.[82] Most importantly, for treatment of diseases such as atherosclerosis, peripheral tolerance can be induced to treat the disease. In atherosclerosis, several strategies to expand Tregs for example by treatment with oral anti-CD3 or IL-2/IL-2 antibody complexes have been shown to reduce atherosclerosis in hypercholesterolemic mice[121,176]. However, these strategies induce an overall expansion of Tregs and possibly also systemic immunosuppression, which is not preferable for a patient. The fundamental aim would instead be to increase the levels of Tregs specific for atherosclerosis related antigens. One approach is by transfer of tolerogenic DCs pulsed with ApoB100 in the presence of IL-10. This was shown to protect against atherosclerosis by dampening of T cell responses to ApoB100[177].

Mucosal tolerance

Another strategy to induce peripheral tolerance is to provoke mucosal tolerance to an atherosclerosis-related antigen. The mucous membranes covering the gut and airways contain a highly specialized mucosal immune system that repeatedly encounter many types of antigens *e.g.* food proteins and antigens derived from the microbiota. Such antigens should not induce an immune response and therefore, the default for non-pathogenic antigens in the mucosa is to generate a Treg response and immune suppression. Induction of mucosal tolerance has been repeatedly shown to treat experimental autoimmune diseases as well as atherosclerosis.[178] Administration of atherosclerosis-related antigens such as oxLDL, HSP60/65 or a combination of ApoB and HSP60 peptides via the oral or nasal route was shown to inhibit atherosclerosis development via the induction of antigen-specific Tregs[179-182]. Mucosal tolerance has been shown to be dependent on TGFβ and the induction of mTGFβ/LAP expression on T cells and B cells[152,183,184].

Coupling of the relevant antigen to cholera toxin B subunit (CTB) potentiates the efficacy of the mucosal tolerance. CTB has been shown to bind to GM1 ganglioside receptors which facilitate the antigen transport across the mucosal barrier and increase uptake and presentation by APC 100-fold[185]. CTB fused to p210 delivered via the intranasal route has been shown to reduce atherosclerosis via the generation of antigen specific Tregs[186]. Additionally, B cells have been reported to be an important tolerogenic APC in mucosal tolerance[184]. Treating B cells with an antigen coupled to CTB induced a Breg phenotype that could turn T cells into antigen specific Tregs. Moreover, transfer of B cells pulsed with myelin oligodendrocyte glycoprotein (MOG), known to be an important antigen in experimental autoimmune encephalomyelitis (EAE), coupled to CTB inhibit the development of EAE[152].

Other immune modulating treatment strategies

Except for immunization strategies using atherosclerosis-related antigens, many other targets for modulating the immune response in atherosclerosis have been investigated.

Targeting co-stimulatory pathways

As mentioned previously, co-stimulatory pathways are of great importance during the induction phase of an immune response. Depending on the type of co-stimulatory molecules that are present on the cells, immune suppression or immune activation is generated. Here, I will mention three pathways that have been shown to be important in atherosclerosis and may be of potential for a future therapy.

CD40-CD40L is a co-stimulatory pathway present on many different types of immune cells as well as SMCs, ECs and platelets. CD40-CD40L interaction signals downstream via TNF receptor associated factor (TRAF) molecules and results in cell proliferation, differentiation and activation.[187] Atherosclerotic mice deficient in CD40 develop reduced amount of disease and increased plaque stability[188] and in accordance, treatment with a CD40L-blocking antibody imitate those results[189]. Unfortunately, prolonged administration of antibodies targeting CD40L have reported increased thromboembolic complications in clinical trials due to the effect of CD40L on platelets[190]. Targeting a specific TRAF molecule (TRAF6) downstream of CD40 while leaving the other TRAF pathways intact reduced the development of atherosclerosis[188]. The same effect was not observed when other TRAF pathways were repressed. This indicates that specifically targeting the downstream signaling molecule involved in the pathogenic effect would possibly reduce the side effects and function as a new therapeutic opportunity.

Another co-stimulatory pathway of importance in atherosclerosis is OX40-OX40L. This pathway has been shown to be of particular importance for long-lasting T cell reaction and inhibits Treg responses[191,192]. Disrupting the interaction between OX40 and OX40L results in decreased atherosclerosis development and can induce plaque regression[193,194].

In contrast to the immune stimulating pathways described above, co-inhibitory pathways are of great importance in maintaining T cell self-tolerance. One example is the PD-PD-L pathway. PD-L1 and PD-L2 is expressed on APC while the PD-1 has a wide distribution both on tissue cells and on T cells.[192] In the absence of both PD-L1 and 2 on an *LDLr*$^{-/-}$ background, mice develop increased systemic immune responses as well as increased amount of atherosclerosis[195].

The pleiotropic effects of statins

Statins is a group of drugs that inhibit 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in the cholesterol biosynthesis pathway. The lipid lowering effect of statins has been proved clinically beneficial for reducing CVD in several studies.[196] However, statins has also been reported to have pleiotropic effects that were also shown to be beneficial for CVD. For example, statin treatment has been shown to improve endothelial dysfunction and to have antioxidant as well as anti-inflammatory properties[197]. In particular, statins affect T cell differentiation into Tregs and has been shown to increase the accumulation of Tregs in the plaque[198-200]. Moreover, statin treatment reduce C-reactive protein (CRP) levels and decrease the expression of adhesion molecules further contributing to the anti-inflammatory property[197,201].

Inhibiting pro-inflammatory cytokines

The role of almost all cytokines has been investigated in experimental atherosclerosis. In general, Th1 related cytokines propagate disease development while Th2 cytokines have been assigned both beneficial and pathogenic roles.

In particular, IL-1β, a classical pro-inflammatory cytokine that also is released following the activation of the inflammasome, has received a lot of attention. Mice genetically deficient in IL-1β or treated with an inhibitory antibody develop reduced amount of atherosclerosis[202,203]. A humanized monoclonal antibody inhibiting IL-1β (Canakinumab) is already approved for treatment of autoimmune disorders and is now evaluated in a clinical trial for CVD.[204] The trial is known as the "Canakinumab Anti-inflammatory Thrombosis Outcomes Study" (CANTOS) and designed to test if inhibition of IL-1β will reduce major cardiovascular events in patients with preexisting CVD[205]. This trial will hopefully be able to answer if inhibiting general inflammation is beneficial in CVD.

IL-22 - bridging immunity and repair

IL-22, a member of the IL-10 related cytokine family, was first discovered in the mouse as IL-10-related T cell-inducible factor (IL-TIF) in 1999[206]. The human orthologue was discovered one year later and the cytokine was then named IL-22[207]. IL-22 share 23% similarity with IL-10 but the two cytokines has been shown to have diverse effects[207]. For example, it was shown that IL-22 stimulation of human monocytes did not decrease the production of TNFα in the same manner as IL-10[207].

Production of IL-22

Activated T cells[207], Th17 cells[208], and a subpopulation identified in humans, Th22 cells[209], produce IL-22 while other leukocytes, macrophages and non-hematopoietic cells do not[210,211]. For some time, there was a confusion regarding the nomenclature for IL-22 producing innate cells. A new cell population was proposed, expressing features of both lymphoid tissue inducer (LTi) cells ($CD127^+ROR\gamma t^+$) as well as NK cell markers (NKp46). The cells were shown to be present mainly in mucosa and to produce IL-22, but not IL-17, in response to IL-23[212]. It is now established that this cell population of IL-22 producing non-T non-B $CD127^+ROR\gamma t^+$ cells are part of a population of cells known as ILCs[213]. Three different groups of ILCs have been described: the group 1 ILCs (ILC1) produce Th1 cytokines such as IFNγ while ILC2s produce Th2-cytokines (IL-5 and IL-13). The third group, ILC3s, include IL-22 producing ILC3s ($NKp46^+$ and $NKp46^-$) as well as LTi cells[214-219].

In T cells, the production of IL-22 is regulated by IL-1β, IL-6, IL-23 and the aryl hydrocarbon receptor (AHR) while TGFβ in contrast to stimulate production of IL-17, inhibits the production of IL-22[220,221].

The IL-22 receptor

As mentioned above, many different immune cell populations produce IL-22. However, the functional receptor for IL-22 (IL-22R) is not expressed on cells of hematopoietic origin and IL-22 has therefore been suggested to be a mediator secreted by the immune system to control tissue responses[210,222,223]. The IL-22R consists of two subunits, the IL-10R2, which is ubiquitously expressed, and the IL-22RA1 subunit with a more limited expression pattern[210,222]. Cells in the intestine, airway, pancreas, skin, liver and kidney have been shown to express the IL-22RA1 subunit that upon ligation signal via JAK/STAT, ERK and JNK dependent pathways[224-226]. Interestingly, there is also a decoy receptor for IL-22

named the IL-22 binding protein (IL-22BP) or IL-22RA2[227,228]. IL-22BP binds IL-22 and blocks the interaction between IL-22 the IL-22 receptor and thus inhibits its activity.

IL-22 in disease

IL-22 has been studied in numerous diseases from autoimmunity to microbial host defense (for review, I recommend reference [229] and [230]). The levels of IL-22 has been shown to be increased in unstable compared to stable carotid plaques and patients with acute coronary syndrome (ACS) have been reported to have increased frequency of circulating Th22 cells[231,232]. However, the direct function of IL-22 in the vasculature is unknown[*]. In this section, I will briefly mention three disease areas where the role of IL-22 has been extensively studied and also includes biological mechanisms that are of relevance for Paper IV in this thesis. Also, the upcoming section highlights that IL-22 has both pro- and anti-inflammatory effects, depending on the tissue and disease context.

Psoriasis

Psoriasis is characterized by thickened epidermis, hyperproliferation and infiltration of immune cells. IL-22 mRNA has been shown to be upregulated in psoriatic skin and higher levels of circulating IL-22 is found in patients with psoriasis compared to controls[233]. IL-22 upregulate the expression of PDGF, MMP1, β-defensins and S100A7-A9 proteins, including psoriasin, in keratinocytes and lung epithelial cells resulting in increased proliferation and migration as well as inhibition of cell differentiation[234,235].

Airway inflammation

Asthma is a chronic Th2 mediated disease characterized by eosinophil infiltration and mucus hypersecretion. In the lung, both lung epithelial cells and airway SMCs express the IL-22 receptor and IL-22 is found in the bronchoalveolar lavage fluid of murine asthma models[210,236,237]. IL-22 has been shown to diminish allergic lung inflammation through the decrease of Th2-promoting cytokine production by the epithelial cells resulting in reduced eosinophil recruitment[238]. Moreover, it was recently reported that human pulmonary SMCs express the IL-22R and respond to IL-22 with increased proliferation and migration[239,240]. Taken together, this suggests that IL-22 protects against the development of asthma but excessive

[*] For further reading about IL-22 and atherosclerosis, see Paper IV in this thesis where I attempt to explain the role of IL-22 in experimental atherosclerosis.

levels could also promote airway remodeling, another pathological feature in asthma.

Diabetes and obesity

Increasing evidence suggests an important role for the immune system in regulating metabolism and thus also metabolic diseases such as T2D[71]. Recently, IL-22 has received a lot of attention in the field of obesity and diabetes, in particular for its role in regulating metabolism homeostasis.

The pancreas is one of the tissues with the highest IL-22R expression. Subjects suffering from obesity or T2D have been reported to have increased frequency of circulating Th22 cells[241]. Moreover, two groups recently reported that treatment with IL-22 could restore insulin sensitivity in obese mice[242,243]. This was accompanied by a direct effect of IL-22 in the protection against endoplasmic reticulum stress as well as in restoring insulin production and storage in the pancreatic beta cells. Interestingly, administration of IL-22 decreased the body weight of obese mice partly by increasing the production of the appetite-reducing peptide YY[242]. IL-22 was also shown to regulate lipid metabolism in the liver and adipose tissue by enhancing lipolysis and fatty acid oxidation.[244]

Methods

Mouse models

In this thesis, I have had the opportunity to study both human and mouse tissue. The ultimate goal would be to test all hypotheses in the human situation but before that is possible, the research community needs a fast and convenient approach to study atherosclerosis development. For this, animal models have been invaluable. Already in 1908 it was shown that rabbits fed animal protein developed atherosclerotic plaques and rabbits were for a long time the primary animal model for studying the disease[245]. Today, the predominantly used species is rodents and the mouse in particular. Interestingly, wild type mice are highly resistant to atherosclerosis because of their lipoprotein profile consisting of high HDL and low LDL levels[246]. Furthermore, mice lack the cholesteryl ester transferase enzyme that transfers cholesterol esters from HDL to VLDL and LDL[246]. This was shown to be strain dependent. The C57Bl/6 strain was shown to be the most susceptible mouse strain and is therefore used as the background for all the atherosclerotic mouse models discussed in this section[247]. Both the *Apoe*$^{-/-}$ and *LDLr*$^{-/-}$ mice develop atherosclerotic plaques in the aorta, aortic arch and in the subvalvular region.

Diet

It was discovered that C57Bl/6 mice could develop atherosclerosis when fed a very cholesterol rich diet, containing 30% fat and 5% cholesterol[246]. However, that diet also contained 2% cholic acid, which was shown to be highly toxic, and the plaque that developed did not resemble the human situation. The cholic acid rich diet was then modified by Paigen and colleagues to yield the "Paigen diet" containing 5% fat, 1.25% cholesterol, and 0.5% cholic acid[247]. The disadvantage with this diet was that the plaques developed slowly and rarely grew larger than fatty streaks. Moreover, the diet was shown to be pro-inflammatory due to the cholic acid content[246]. A more physiological diet, based on the typical American diet containing 21% fat, 0.15% cholesterol and importantly, no cholic acid, was then developed[248]. This diet was assigned a "western diet" or as in our studies, a "high fat diet" (HFD), and is frequently used in combination with genetically modified strains of mice to study atherosclerosis development.

ApoE deficient mice

ApoE is a structural component of almost all lipoproteins (except of LDL). ApoE binds to the LDLr on hepatocytes and mediates the clearance of lipoproteins from the circulation[249]. Generation of the ApoE deficient mice was a major breakthrough for atherosclerosis research[248,250]. The ApoE deficient mice develop a five-time increase in total cholesterol levels on a chow diet and 15-18-fold increase on a western diet. In particular, HDL levels in this model is reduced with 55% and accompanied by a shift from HDL to CM remnants and VLDL, while the triglyceride level is increased with 68%. The mouse model develops atherosclerotic plaques already when fed chow but plaque progression is impressively enhanced on a western-type diet. Remarkably, the plaques that these mice develop are similar to the human phenotype[251]. The ApoE deficient mouse model is used in paper I and IV.

LDLr deficient mice

LDLr is the expressed on hepatocytes and involved in clearance of lipoproteins from the circulation. The LDLr deficient mouse was created in 1993 to resemble familiar hypercholesterolemia[252]. The increase in LDL and VLDL levels are milder in these mice compared to the ApoE deficient mouse model and the LDLr deficient mice do not develop plaques on a chow diet but are highly responsive to a western diet. The LDLr deficient mouse model is to prefer when performing bone marrow transfer experiments since bone marrow cells can produce ApoE and therefore restore hyperlipidemia if transferred into *Apoe*$^{-/-}$ mice[253].

ApoB100 transgenic mice

As mentioned previously, humans express ApoB48 in the intestine and ApoB100 in the liver while mice express ApoB48 both in the intestine and in the liver[11]. Although mice still produce ApoB100 in the liver, the limited ApoB100 content may be a drawback when studying adaptive immune responses against ApoB100 derived peptides. There are several mouse models handling with this issue in different ways. In paper II we used a mouse model where a point mutation is introduced in the ApoB48 editing codon of the ApoB gene, generating a mouse model that only synthesize ApoB100.

The ovalbumin transgenic mouse model

The ovalbumin (OVA) transgenic mouse, known as the OT-II mouse, expresses a transgenic MHCII-restricted, OVA-specific TCR. In specific, the majority of the $CD4^+$ T cells in this mouse model recognize amino acids 323-339 of OVA (pOVA). The OT-II mouse was developed 1998 and has since then been used for several studies regarding thymic selection, TCR interactions and tolerance[254]. This mouse model was used in paper II to study the induction of antigen specific Tregs. It should be noted that the mouse model used in paper III still are able to develop mature T cells expressing endogenous TCRs meaning that not 100% of all $CD4^+$ T cells express the transgenic TCR.

Matrigel

To be able to study ongoing antigen specific immune responses *in vivo*, we[†] adapted a method that was first developed by Corthay and colleagues[255] to study immune responses against tumor cells. Matrigel is composed of ECM proteins such as collagen IV, laminin and perlecan, derived from the Engelbreth-Holm-Swarm tumor[256]. The interesting feature about matrigel is that it appears as a viscous fluid at low temperature (around 4°C) while matrigel forms a solid gel at body temperature.

In brief, in paper I we aimed to utilize the matrigel-model to study antigen-specific Th2 responses targeting ApoB100 and its role in atherosclerosis. HFD fed *Apoe*$^{-/-}$ mice were immunized with ApoB100 in Alum, Alum only or left untreated. One week after the last immunization matrigel, mixed with ApoB100 or control buffer, were injected subcutaneously on each flank of the mice, generating an intrinsic control. After seven days, we retrieved and enzymatically digested the matrigel plugs to study the infiltrating immune cells by flow cytometry and measure the release of cytokines.

[†] The matrigel-method used in paper I have previously been extensively described in the thesis by D Engelbertsen, *Adaptive Immunity in Cardiovascular Disease*. ISBN 978-91-87449-21-5.

The underlying series of events explaining this method are first, that the insults of injecting matrigel in it self trigger influx of monocytes (illustrated in Figure 3). Monocytes that enter the plug will phagocytize and present antigen on MHCII to trafficking $CD4^+$ T cells. $CD4^+$ T cells that recognize such an MHCII-peptide complex will produce effector cytokines and upregulate CD25, the alpha-subunit of the IL-2 receptor, in response to activation. This will further recruit more leukocytes leading to accumulation of cells specific for the particular antigen found in the matrigel plug.

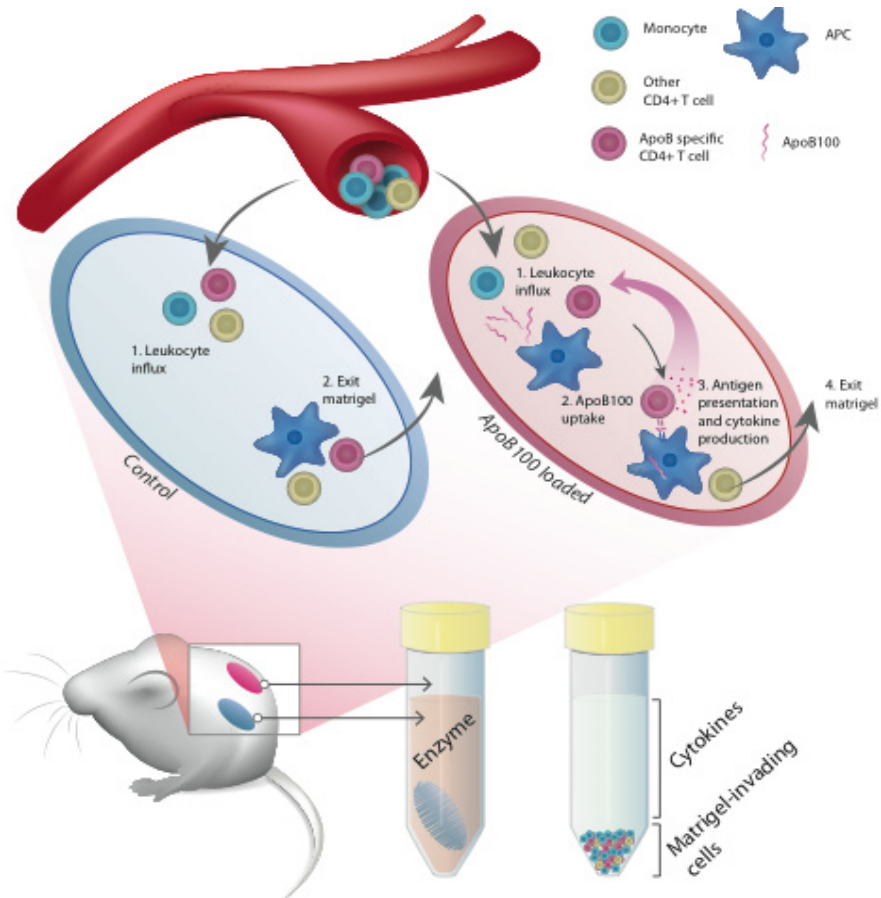

Figure 3. Schematic picture illustrating the matrigel method

Populations studies

Mouse models are excellent tools for biomedical research but the ultimate goal is to validate experimental findings in humans. In paper III and V, I have utilized three separate cohorts to be able to test the clinical usefulness of the two topics in my thesis. In this section, I will first describe the three cohorts and then briefly discuss my view on statistics.

The SUMMIT study

The SUrrogate markers for Micro- and Macrovascular hard endpoints for Innovative diabetes Tools (SUMMIT) study is a prospective case-control study with the aim to identify markers for micro- and macrovascular complications in diabetes. The study cohort contains four patient groups; T2D subjects with and without prevalent CVD as well as non-T2D with and without CVD. CVD includes non-fatal acute MI, hospitalized unstable angina, resuscitated cardiac arrest, any coronary revascularization procedure, non-fatal stroke, transient ischemic attack confirmed by a specialist and lower extremity arterial disease. At the basal examination, several clinical parameters were recorded such as ultrasound measurements of carotid intima-media thickness (IMT) and reactive hyperemia index to assess endothelial dysfunction. In paper III, we randomly selected approximately 50 patients recruited in Malmö, Sweden, from each patient group and measured the levels of circulating Tregs in blood. We analyzed the percentage of $CD25^{+}CD127^{dim}$, $CD25^{+}FoxP3^{+}$ or $FoxP3^{+}$ out of $CD3^{+}CD4^{+}$ cells or as cell number per µl blood as well as utilized Helios to distinguish two subpopulations of Tregs.

The Malmö Diet and Cancer Study

The Malmö Diet and Cancer (MDC) study is a population-based, prospective, epidemiological cohort including 28 449 persons enrolled between 1991 and 1996[257]. The included subjects were born between 1926 and 1994 and lived in Malmö by the time of inclusion. From the MDC cohort, 6103 persons were invited to participate in a substudy, the MDC Cardiovascular Cohort, which was designed to investigate the etiology of carotid artery disease. At baseline examination, fasting plasma samples were taken and stored at -80°C until analyzed during spring 2014. During a median follow-up time of 15.4 years, 384 patients suffered from a first-incident CE. In paper V, the levels of three SMC growth factors in plasma (PDGF, HB-EGF and EGF) were measured by a Proximity Extension

Assay (PEA) technique using the Proseek Multiplex CVD96x96 reagents kit in 793 subjects[258].

The Carotid Plaque Imaging Project

Human plaque tissue was acquired from the Carotid Plaque Imaging Project (CPIP). This biobank consists of plaques collected from patients undergoing carotid endarterectomy at the Vascular Department of Skåne University Hospital in Malmö. The criteria for surgery were ipsilateral symptoms and a stenosis greater then 70% ("symptomatic") or a stenosis larger then 80% for patients without symptoms ("asymptomatic"). The degree of stenosis was assessed using ultrasound. Plaques from patients with *amaurosis fugax,* transient ischemic attack or stroke were considered symptomatic. Information regarding post-operative cardiovascular events was gathered through the Swedish national register of hospitalizations and telephone interviews.[259]

Plaque tissue was directly snap-frozen in liquid nitrogen. After surgery, one-millimeter thick portion from the most stenotic part of the plaque was saved for histological analyzes. The remainder of the plaque was homogenized for analyzes of plaque components[259]. In paper V, we compared histological quantification of lipids (Oil-Red-O staining), SMCs (α-actin staining) and macrophages (CD68 staining) as well as total collagen and elastin content, measured in plaque homogenate using biochemical techniques. Also, the levels of several MMPs as well as caspase 3 activity were measured in plaque homogenate using multiplex techniques and ELISA, respectively. Data was normalized to plaque wet-weight.

One day before the surgery, blood samples were taken and plasma was stored at -80^{o}C until analyzed. During the spring of 2014, the plasma levels of three SMC growth factors (PDGF, EGF, HB-EGF) were measured in 202 patients by a PEA technique[258].

Statistics

It is possible to write numerous books focusing entirely on statistics. With that said, I will only briefly mention some of the statistical methods used in the papers included in this thesis.

In all papers presented, statistical methods have been applied to evaluate the findings and to compare treatment effects. Statistical tests generate a p-value, which denotes the likeliness of the difference being a consequence of chance. Most often, the cut-off for rejecting the null-hypothesis (*i.e.* there is no difference between the groups) is $p<0.05$ which means that 5% of the performed analysis will generate a statistically significant outcome without a true relationship.

In common for both paper III and V, the design of the studies only allows us to look at correlations. Importantly, significant correlations do not automatically imply causality. Some correlations give valuable information that might be considered as causality but it is essential to know when the information is important for the outcome (*e.g.* the disease event) or not. This issue is nicely illustrated in a study using the familiar folk tale that storks deliver babies as the starting point[260]. They aimed to investigate if there is a correlation between the number of storks in a country and the number of human childbirths in the same country. Results revealed a highly significant correlation, which can be statistically interpreted as "storks deliver babies" but which apparently is causally irrational. Probably, there is a confounding factor in this study that affects both variables in the equation (*e.g.* size of the country). This type of outcome is very common in studies where the clinical endpoint already have many well-established underlying risk factors (confounders). Therefore, correlations should be interpreted with caution and there are statistical methods that can take confounders into account when performing the analysis. In both paper III and V, the associations were adjusted for confounding factors to be able to imply an independent relationship between the variables.

More detailed description regarding the statistical methods can be found in the method section in the respective paper.

Aims and Key Findings

Paper I

Aim: To develop and evaluate a method to study antigen-specific immune responses *in vivo* and to investigate if induction of a Th2 immune response against human ApoB100 affects atherosclerosis development in *Apoe*$^{-/-}$ mice.

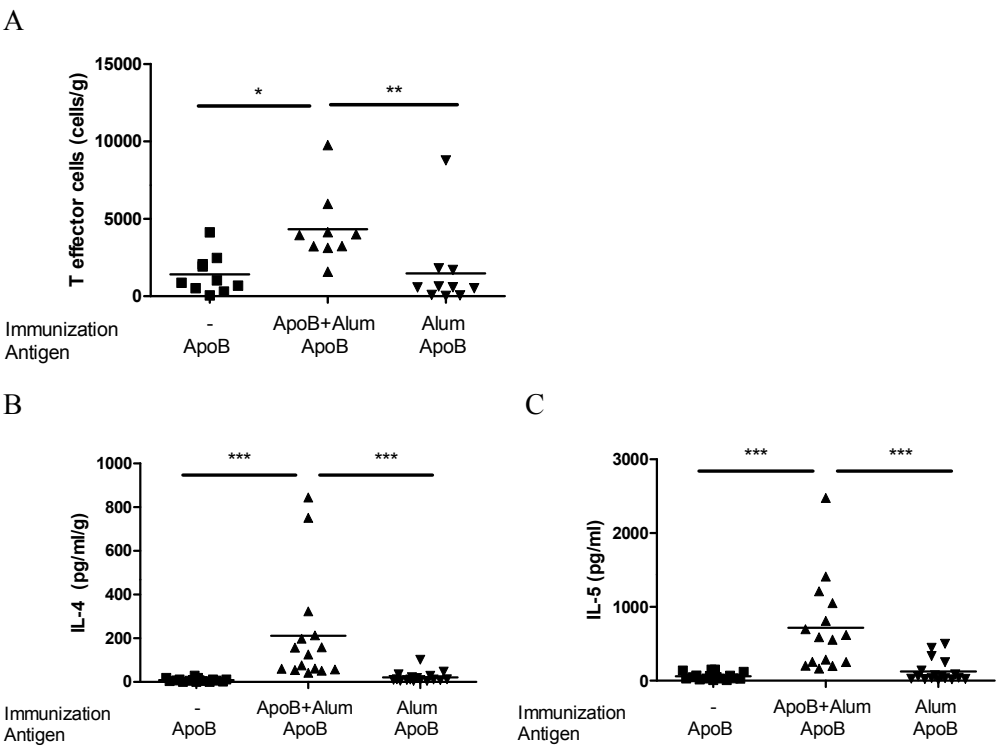

Figure 4. Increased accumulation of T effector cells (A) and increased production of Th2 related cytokines (B-C) in the ApoB100 loaded matrigel plug after ApoB100 immunization.

Key findings: Using the matrigel system (Figure 3), we could detect a Th2 immune response against human ApoB100 in response to immunization with human ApoB100 (administered with Alum as the adjuvant) compared to unimmunized mice or Alum alone. This was demonstrated by an accumulation of T effector cells and the release of Th2 cytokines in ApoB100-loaded matrigel plugs after ApoB100 immunization (Figure 4). Also, ApoB100 immunized mice developed antibodies towards both mouse and human LDL. However, the induction of a Th2 response against human ApoB100 did not affect atherosclerosis development.

Paper II

Aim: To determine if p210 coupled to CTB can induce functionally active Bregs *in vitro* and to investigate if transfer of p210-pulsed Bregs can affect atherosclerosis development.

Key findings: B cells pulsed with p210-CTB, expressed Breg markers (mTGFβ/LAP) and induced generation of Tregs *in vitro* (Figure 5). Transfer of p210-CTB pulsed Bregs into hypercholesterolemic mice did not transfer the tolerogenic effect observed *in vitro* nor did it affect atherosclerosis development. However, this might be explained by the induction of an immune rejection response against the male B cells in the female recipients.

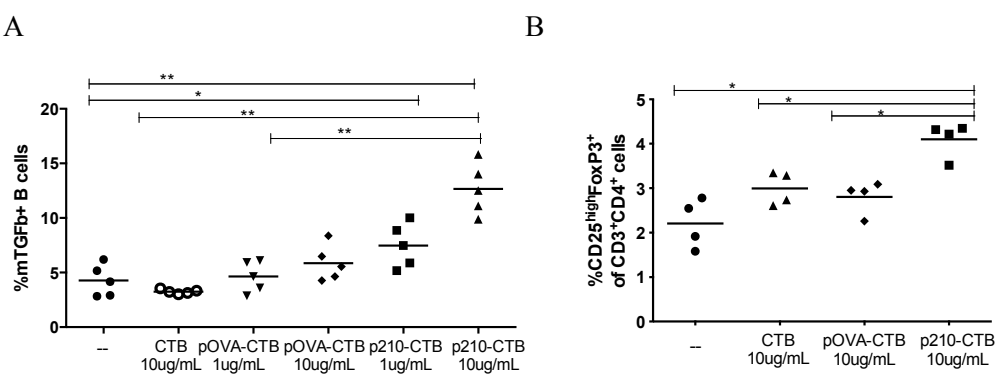

Figure 5. p210-CTB pulsed B cells upregulate mTGFβ/LAP (A) and induce Tregs (B) *in vitro*.

Paper III

Aim: To study Treg levels in patients with T2D and/or CVD. Can lower Treg levels explain the increased risk for CVD in patients with T2D?

Key findings: Unexpectedly, patients with CVD, regardless of T2D, were shown to have a higher percentage of circulating Tregs (Figure 6). This could partly, but not entirely, be explained by the use of statins. Percentage Tregs were positively associated to T effector cells indicating that Treg levels can increase in response to the general immune cell activation that occur in patients with CVD.

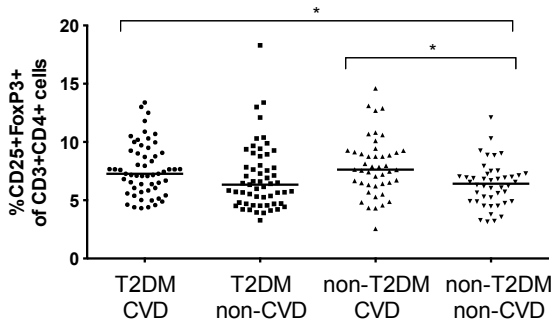

Figure 6. Increased percentage of circulating Tregs in patients with CVD, irregardless of T2D.

Paper IV

Aim: To elucidate if IL-22 affects atherosclerosis development.

Key findings: *IL-22*$^{-/-}$*Apoe*$^{-/-}$ mice were characterized by reduced development of atherosclerosis and this was associated with increased expression of contractile SMC markers in the carotid artery and in the medial layer of the aortic root as well as lower collagen content in the plaques (Figure 7). Moreover, IL-22 treatment of mouse aortic SMCs resulted in decreased expression of contractile genes. We conclude that IL-22 affects SMC switching into a more synthetic phenotype and that reduced numbers of SMCs synthesizing collagen in IL-22 deficient mice may contribute to the reduced plaque size.

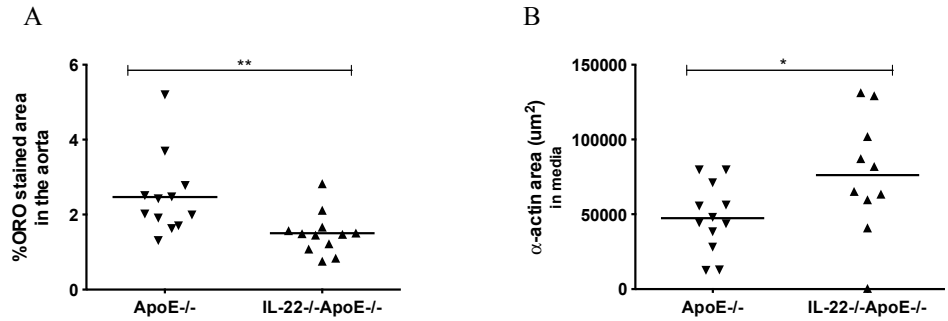

Figure 7. Decreased plaque size (A) and increased amount of medial α-actin (B) in IL-22 deficient *Apoe*$^{-/-}$ mice.

Paper V

Aim: To investigate if SMC growth factors measured in plasma can reflect the plaque phenotype and predict incidence of CE in a general population.

Key finding: The levels of PDGF, HB-EGF and EGF in plasma were associated with the collagen and elastin content of carotid plaques. A high level of HB-EGF at baseline was associated with a lower risk of developing a CE in the general population (Figure 8).

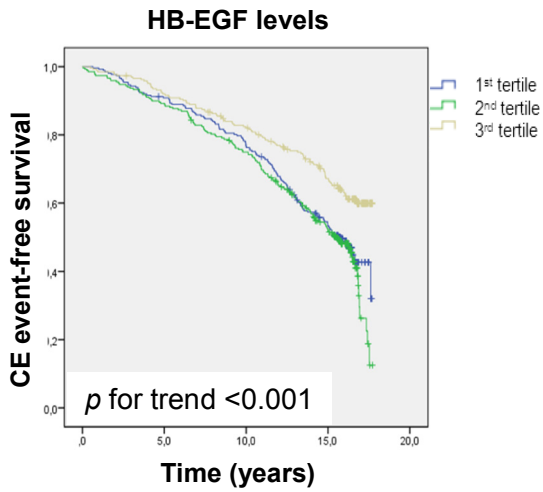

Figure 8. Association between baseline plasma HB-EGF levels and incidence of CE.

Results and Discussion

Regulatory immune responses

Th2 immunity in atherosclerosis

In paper I, we developed a matrigel-based method to study antigen specific immune responses in atherosclerosis (described in methods and illustrated in Figure 3). After immunization with human ApoB100 in Alum we could detect an accumulation of T effector cells in matrigel plugs containing ApoB100 but not in control plugs (containing vehicle) or after immunization with only Alum. Additionally, we observed production of Th2-related cytokines locally in the ApoB100-containing plug as well as increased levels of ApoB100 specific antibodies in plasma in mice immunized with ApoB100 (Figure 4). Even though we induced an active ongoing Th2 immune response against human ApoB100, both humoral and cellular, no effect was observed on the amount of atherosclerosis.

Based on paper I, it cannot be excluded that Th2 immunity play an important role in atherosclerosis development. We show that immunization with human ApoB100 in Alum generates a Th2 response, both locally in the matrigel as well as systemically, by the production of antibodies targeting human ApoB100.

Th2 responses have been suggested to have an overall protective role in atherosclerosis but the evidence is limited and inconsistent results have been reported. The evidence is mainly based on the fact that mice lacking Th1 immunity (IFNγ- or T-bet-deficient mice) should have a compensatory increase of Th2 responses, thus resulting in reduced atherosclerosis. Other evidence comes from studies showing that immunization with MDA-LDL expanded the levels of IL-5 secreting Th2 cells resulting in increased levels of protective natural IgM antibodies and decreased atherosclerosis[261]. In turn, bone marrow transfer of IL-5 deficient cells led to decreased titers of those antibodies and increased amount of atherosclerosis[261]. Hence, stimulating B cells to produce antibodies is one important function of Th2 cells in the protection against atherosclerosis. In paper I, we observed increased production of Th2 isotype switched antibodies targeting human ApoB100 but we were also able to generate an antibodies that cross-react with mouse ApoB100. However, the cross-reaction with mouse ApoB100 was

substantially lower compared to the antibody response generated to human ApoB100. If antibodies are the main mechanism by which Th2 immunity against ApoB100 affects atherosclerosis, the considerably lower cross-reactivity between human and mouse ApoB100 in this study may have influenced the lack of effect on atherosclerosis.

Another Th2-related cytokine, IL-4, have been shown to induce a M2-polarized macrophage phenotype and thereby possibly contributing to plaque stabilization[262]. In the present study, no differences were observed for plaque size or for plaque stability after induction of a Th2 response against human ApoB100. Other possible interpretations could be either that the induced immune response was not efficient enough to affect the disease or that ApoB100 is not an important antigen in atherosclerosis (as discussed later) when targeted by a Th2 response.

We observed increased production of cytokines previously mainly related to a Th2 response (IL-4, IL-5) but more recently, ILC2s have been shown to be a great source of Th2 associated cytokines. However, we were also able to detect an increased IL-4 production in T cells in the ApoB100 loaded plugs compared to vehicle controls, after ApoB100 immunization. This suggests that T cells are the main source of Th2 cytokines in our study but we cannot exclude that other cell populations also contribute to the production of cytokines in the plug.

Transfer of p210-CTB pulsed B cells to treat atherosclerosis

Induction of mucosal tolerance against atherosclerosis related antigens have previously been utilized to reduce atherosclerosis development. Coupling of the antigen of interest to CTB is known to potentiate the induction of mucosal tolerance when administered orally or nasally. Recently, Bregs have been implicated as an important mediator of mucosal tolerance. Bregs are an interesting immunosuppressive cell population that has not been widely studied in atherosclerosis. Based on the production of IL-10 and the capability to induce Tregs, it is likely that Bregs would act protective in the disease.

In paper II, we adapted a method by Sun *et al* to study if transfer of p210-CTB induced Bregs can affect atherosclerosis development. In brief, Sun and colleagues first established that treatment of B cells with OVA-CTB *in vitro* induced a Breg phenotype with mTGFβ/LAP expression. These Bregs could turn naïve T cells into antigen specific Tregs, when T cells from OT-II mice were used. To test if antigen-CTB pulsed B cells have a regulatory activity *in vivo* and protect against EAE, they pulsed B cells with MOG-CTB and transferred them into mice. Using this approach, mice developed milder EAE and had increased levels of Tregs and decreased T cell proliferation. [152]

We pulsed B cells with p210 coupled to CTB because p210 has been extensively investigated as an important antigen in atherosclerosis. Increased expression of mTGFβ/LAP could be observed on the B cells pulsed with p210-CTB and these cells could induce Tregs after co-culture *in vitro* (Figure 5). However, after transferring p210-CTB pulsed B cells into mice that already had started to develop atherosclerosis, no induction of tolerance (Bregs or Tregs) or affect on atherosclerosis could be observed.

The gender matters

In this study, we transferred male B cells into female recipients without paying attention to the effect of gender on host-versus-graft reactions. We observed increased basal production of Th1/Th2 cytokines as well as increased basal splenocyte proliferation in all mice receiving B cells, independent of B cell treatment. This reflects induction of an immune response rather than tolerance and it became evident that we should have considered the gender of the donor mice before transfer.

It was first demonstrated in mice that skin grafts from males to females had the highest rate of graft rejection[263]. Furthermore, this effect could be prevented if newborn females were tolerized to the male cells, which lead to the identification of the male Y-chromosome-encoded minor histocompatibility antigens (H-Y)[264]. The clinical impact of the H-Y antigens has been intensively studied. For example, it has been demonstrated that female patients receiving a kidney from a male donor have increased risk of transplant rejection and male recipients of female bone marrow have an increased risk for graft-versus-host reactions[263,265,266]. This was explained by an immune reaction where female immune cells recognize the H-Y antigens present on normal male tissues. H-Y antigens are widely distributed and CD8 and CD4 T cell responses as well as antibodies targeting antigens encoded by H-Y have been reported[267-270]. Female recipient mice has been shown to reject male cells 10-12 days after transfer[271].

Taken together, this indicates that the lack of effect of Breg transfer on atherosclerosis and induction of tolerance *in vivo* in paper II may be explained by the activation of a host-versus-graft rejection that masks the potential tolerogenic effects of Breg transfer. In addition, the repeated number of injections may further contribute to the rejection response. Interestingly, the evoked immune response, possibly targeting H-Y antigens, did not increase the amount of atherosclerosis in mice receiving B cells emphasizing the need of antigen-specific immune responses in atherosclerosis.

It may be argued that 10-12 days would be enough for the transferred Bregs to confer its tolerogenic effects. Another possible explanation to the lack of effect on atherosclerosis could be that we transferred B cells pulsed for only one hour with p210-CTB into mice that already had started to develop atherosclerosis. It may be

that a different treatment schedule or selection of the mTGFβ/LAP$^+$ B cells prior to transfer would result in a better effect on atherosclerosis. However, the B cell transfers were performed according to the protocol developed by Sun and colleagues but may be that the method developed to treat EAE is not effective in order to treat other inflammatory disease such as atherosclerosis. In addition to the male-to-female transfer, difference in the route of transfer could have affected the outcome in the present study. In our study, B cells were transferred *intraperitoneal (i.p)* while Sun *et al* used *intravenous (i.v)* injections. A previous study transferring CD8$^+$ T cells showed that the *i.p* route resulted in an increased amount of transferred cells ending up in the spleen compared to the *i.v* route[272]. However, it might be that the *i.v* route is to prefer when aiming to induce tolerance[273].

ApoB100 as an antigen in atherosclerosis

The two first papers presented in this thesis assume that ApoB100 or ApoB100 derived peptides are important autoantigens in atherosclerosis. Both cellular and humoral immunity have been shown to target LDL in atherosclerosis[86,274]. In paper I, we study immune responses to human ApoB100 in ApoE deficient mice. Mice immunized with oxLDL have T and B cell clones that recognize ApoB100 and blocking of the T cell responses to LDL or transfer of tolerogenic DCs pulsed with ApoB100 inhibit atherosclerosis development[169,177]. This indicates that human ApoB100 can be a functionally important antigen in atherosclerotic mice but it is important to note that these studies were performed in transgenic mice expressing human ApoB100.

The obvious limitation in paper I is the use of human ApoB100 as the antigen to study atherosclerosis in ApoE deficient mice. The human ApoB100 sequence is 70% homologous to the mouse counterpart and we were able to detect a cross-reaction between the proteins after immunization with human ApoB100, as assessed by the presence of antibodies targeting both the human and mouse protein. Moreover, T effector cells accumulated in matrigel plugs containing human ApoB100 when implanted in old atherosclerotic *Apoe*$^{-/-}$ mice on HFD but not in young chow fed *Apoe*$^{-/-}$ mice, indicating that atherogenic conditions generates T effector cells that recognize human ApoB100.

ApoB100 is a large protein and for convenience, it would be preferable to know which part of ApoB100 that is targeted by autoimmune reactions in atherosclerosis. In paper II, we used peptide number 210 from ApoB100 as the antigen. The peptide sequence is derived from human ApoB100 and show 90% homology to the mouse sequence[175]. The peptide p210 has been used as the antigen in several immunization studies to treat experimental atherosclerosis. It is unlikely that p210 being "an irrelevant antigen" can fully explain the lack of effect on atherosclerosis after transfer of p210-CTB pulsed B cells in paper II. A

previous study using intranasal administration of p210-CTB reported the induction of antigen-specific Tregs and reduced atherosclerosis development. Moreover, the authors show that Tregs induced by p210-CTB can inhibit antigen (ApoB100)-specific T cell proliferation indicating that immune response to p210 also targets full-length ApoB100. Given the host-versus-graft reaction it is hard to draw any conclusions regarding the effect of p210-CTB-pulsed Bregs on atherosclerosis.

The disadvantages with using small single peptides in vaccine formulations include the fact that MHC molecules among individuals bind different peptide sequences depending on the human leukocyte antigen (HLA) type. This means that some HLA may not present the particular peptide and HLA genotyping may be needed to individualize the peptide vaccine to fit the particular patient. When stimulating human cells with p210-CTB we could detect an increased expression of mTGFβ/LAP on the B cells but no increase in Tregs after co-culture. This may support the idea that the HLA type is of importance for the effect. Also, it has been previously shown that the effect of antigen-CTB fusion proteins is abrogated in mice deficient in MHCII[152]. In addition, our data show that OVA-CTB pulsed B cells can induce Tregs when using OVA-specific T cells but not when cells from *Apoe*$^{-/-}$ mice were used. This indicates that the targeted T cells needs to have specificity for the antigen in order to be converted into a Treg.

As described in the background, other antigens such as HSP60 have also been implicated in the disease. Interestingly, combining ApoB100 and HSP60 peptides in oral tolerance or epitopes from HSP60, ApoB100 and *Chlamydophila pneumonia* had a better inhibitory effect on atherosclerosis development compared to using each epitope alone[182,275]. In addition, subcutaneous infusion of a combination of ApoB100 peptides reduced atherosclerosis development[276]. This suggests that combining several atherosclerosis-related antigens may generate a more diverse immune response targeting several epitopes and hence, have a more pronounced effect on the disease.

Tregs and CVD in man

T2D has become a growing risk factor for CVD and subjects with T2D suffer from a 2-4-fold increased risk for developing CE. T2D has been associated with chronic low-grade inflammation and atherosclerosis is considered an inflammatory disease.

In paper III, we asked if alterations in regulatory immunity, measured as Treg levels, could explain the increased risk for CVD in T2D patients. Therefore, we measured the levels of Tregs in blood from four patient groups; T2D subjects with or without prevalent CVD (n=57 and n=57, respectively) as well as in non-diabetics with or without prevalent CVD (n=45 and n=44, respectively) by flow cytometry. In our study, Tregs were characterized as percentage of $CD25^{+}FoxP3^{+}$,

$FoxP3^+$ or $CD25^+CD127^{dim}$ cells out of $CD4^+CD3^+$ cells or as cell number per µl blood. Surprisingly, we found increased percentage of all three Treg definitions in patients with CVD, irrespective of T2D (Figure 6). No association was observed for percentage Tregs and T2D. On the other hand, when analyzing Treg number per µl blood, subjects with T2D was shown to have decreased numbers of Tregs, irrespective of CVD.

In mice, Tregs are defined as $CD4^+FoxP3^+CD25^+$ T cells while the human Treg population is more heterogeneous[277]. The transcription factor FoxP3 is considered the master regulator of Treg differentiation but activated T cells have been shown to transiently upregulate FoxP3[278]. Except for the expression of FoxP3 and CD25, Tregs have been reported to have a diminished level of CD127 (the IL-7 receptor). The expression of CD127 was shown to be inversely associated with the suppressive function and the expression of FoxP3 and therefore considered a good surface marker for human Tregs[113,279]. In paper III, we used three markers to generally define Tregs; CD25, FoxP3 and CD127. All three Treg populations ($CD25^+FoxP3^+$, $FoxP3^+$ and $CD25^+CD127^{dim}$) correlated strongly to each other and gave the same results in our cohort, indicating that they comprise a similar cell population.

Why do patients with prevalent CVD have increased percentage of Tregs?

Our observation of increased percentages of Tregs in blood from patients with prevalent CVD is contradictory to some previous studies. This may be due to difficulties in defining human Tregs, quality of flow cytometry data as well as differences in patient characterization and blood sampling time point, between the different studies.

In line with a multitude of experimental studies pointing towards a protective role for Tregs in atherosclerosis, results from a prospective cohort reported an association between high levels of circulating $FoxP3^+CD4^+$ Tregs and a lower risk of incident CE. Moreover, it has been shown that patients with ACS (including angina, STEMI and non-STEMI) have lower levels of $CD4^+CD25^+$ and $CD25^+FoxP3^+$ Tregs compared to healthy subjects[123,124].

Our study differs from the above-mentioned studies in two major ways. First, we used a broader definition of CVD (including non-fatal acute MI, unstable angina, cardiac arrest, any coronary revascularization procedure, non-fatal stroke, transient ischemic attack and lower extremity arterial disease). The involvement of Treg levels may differ depending on the type of CVD. This was further signified in two studies analyzing patients with non-STEMI and STEMI separately. It was reported that patients with non-STEMI had lower levels of Tregs ($CD4^+CD25^+$ or $CD4^+CD25^+CD127^{dim}$) compared to controls (healthy subjects or when compared to a group of patients with chest pain but without coronary stenosis) while STEMI patients had higher levels of Tregs compared to controls[127,280]. Together with our

results, this indicates that the involvement of Tregs in CVD is more complex than first anticipated. Secondly, previous studies analyzed the Treg levels in patients admitted to the intensive care presenting with ACS. In our study, the blood samples were not taken during the acute phase but rather several years after the clinical event. Interestingly, Ammirati *et al*. observed increased Treg levels in both STEMI and non-STEMI patients 55 days after the CE compared to the first measurement supporting the idea that differences in the time of analysis may at least partly explain the conflicting results among the clinical studies investigating Treg levels in CVD[127].

What is the explanation for the increased percentage of Tregs in patients with prevalent CVD? Except for lowering cholesterol levels, statin treatment have been shown to improve endothelial dysfunction and to have antioxidant as well as anti-inflammatory properties[197]. In particular, statins affect T cell differentiation into Tregs and has been shown to increase the accumulation of Tregs in the plaque[198-200]. Moreover, statin treatment has previously been shown to increase the levels of Tregs in patients with STEMI[199]. In accordance, we observed increased frequency of Tregs in patients treated with statins. In our cohort, 82.4% of all the CVD patients are treated with statins and this is likely to have contributed to the increased levels of Tregs in patients with prevalent CVD. However, this was not solely an effect of statins because the association between Tregs and CVD remained significant after adjusting for lipid lowering drugs and CVD risk factors.

Patients with CE have been shown to have an acute increase in activated T cells ($CD69^+$) and IFNγ producing Th1 effector cells[124,127]. As mentioned above, the levels of Tregs were shown to continue to increase up to 55 days after the CE while the levels of activated T cells were stable in these patients[127]. In paper III we report a strong positive correlation between Tregs and $CD4^+$ T effector cells ($CD62L^-CD45RO^+$) while Tregs were negatively associated to naïve T cells ($CD4^+CD62L^+CD45RO^-$). Furthermore, unpublished analysis from this cohort shows a trend towards increased frequency of T effector cells in patients with CVD (p=0.057, D. Engelbertsen *et al.*, unpublished results). In line with our results, increased levels of FoxP3 have been demonstrated in vulnerable compared to stable plaques suggesting that anti-inflammatory mediators can accumulate in response to inflammation[281,282]. Taken together, this supports the hypothesis that Treg levels increase in patients with CVD as a compensatory mechanism to dampen the immune response. However, it should also be noted that genetic factors or environmental conditions could contribute to increase the levels of Tregs or decrease the levels of other T cell populations in certain subjects, thus making Tregs "guilty-by-association".

Cell percentage versus number

Except for Treg percentages, Treg cell numbers per µl blood was also assessed in paper III. However, we did not observe any difference for Treg number between

CVD cases and controls indicating that the proportion of cell populations, rather than cell numbers, is altered in these patients. On the other hand, patients with T2D had a lower number of Tregs while no difference was observed for percentages. This was at least partly explained by an overall decrease in the number of $CD3^+CD4^+$ T cells in T2D subjects, which was not observed in patients with CVD. One previous study reported significantly lower Treg percentage and cell number in patients with T2D[283]. The same study also showed that T2D patients with microvascular complications (neuropathy, nephropathy or retinopathy) had a lower percentage of Tregs compared to T2D subjects with macrovascular complications (cardiovascular, peripheral vascular or cerebrovascular disease) and when compared to healthy controls, while Treg numbers are not shown[283]. In our study, we did not separate the T2D patients depending on the type of vascular complication due to the limited number of study subjects, but it may be that different types of vascular complications show different patterns of associations in this respect.

The clinical studies investigating the association between Tregs and CVD have mainly reported percentage of Tregs and not cell numbers. This may be because of difficulties in properly assessing cell numbers of different cell populations by flow cytometry. In our study, we used counting-beads to be able to assess the amount of sample that was run in the flow cytometer. By knowing the amount of beads that were added to each sample, the number of cells per µl blood in that sample could be calculated. In addition, for our Treg analysis, $CD4^+$ cells were first enriched from whole blood before staining. This means that another sample with unsorted cells were needed to be able to calculate the concentration of $CD3^+CD4^+$ cells in blood from each patient. This adds to the complexity of considering cell numbers in this cohort.

Is Helios a marker for nTregs?

Part of the novelty in paper III is that the transcription factor Helios was included as a marker in order to distinguish the origin of the Tregs. The source of a given Treg may be important to determine when monitoring new therapeutics that aim to induce Tregs.

Evidence from microarray analysis of Tregs and naïve T cells has indicated that Helios is preferentially expressed in Tregs[116,284]. Furthermore, Helios was shown to be expressed in all $CD4^+CD8^-FoxP3^+$ thymocytes while the expression in the periphery was restricted to approximately 70% of $FoxP3^+$ T cells[117]. Tregs induced *in vitro* or *in vivo* failed to express Helios, suggesting that Helios distinguish nTregs from iTregs[117,285].

In paper III, we did not observe any association between the percentage of $Helios^+$ or $Helios^-$ Tregs among patients with CVD or T2D indicating that Helios is not a discriminating factor for Tregs among these patients. On the other hand, patients

with T2D had a lower number of Tregs, including Helios$^+$ Tregs, while there was no significant difference for Helios$^-$ Tregs. A previous study reported decreased percentage of Helios$^-$ Tregs in T2D patients while no difference was observed for Helios$^+$ Tregs. However, this is based on percentages and cell numbers are not reported[283].

The use of Helios as a marker for nTregs has been questioned. More recent studies have reported that the particular *in vitro* condition used to induce Tregs seem to determine Helios expression rather than cell origin and Helios can be transiently upregulated in activated T cells[118,286]. Additionally, lack of Helios does not exclusively define iTreg since there are Helios$^-$ Tregs in the nTreg compartment[119].

Other limitations

There are a few additional limitations with the present study. First of all, we study Tregs in blood which means that we look at cells in transit at only one time point. This may not reflect the amount of Tregs that are present at the site of inflammation, *i.e.* the plaque. Moreover, we do not study antigen-specificity or suppressive capacity. For example, the suppressive function of Tregs isolated from both hypercholesterolemic mice and patients with CVD have been shown to be compromised indicating that the increase of Tregs in the present study may compensate for the lack of suppressive function[123,128].

Repair mechanisms

IL-22 bridging immunity and repair

Regulation of immune responses is of great importance in plaque development and stabilization. Atherosclerosis can be considered a chronic non-resolving inflammatory disease and understanding the principles of inflammation resolution is vital to understand how the disease progress[23]. In paper IV, we wanted to study if IL-22, a mediator secreted by immune cells, can regulate tissue repair. To do this, we generated an *IL-22$^{-/-}$Apoe$^{-/-}$* double knockout mouse model‡ to study atherosclerosis development. When fed HFD, IL-22 deficient *Apoe$^{-/-}$* mice exhibit reduced plaque size accompanied by increased expression of contractile SMC markers (Figure 7).

‡ Generation of a double knockout mouse model can take tremendous amount of time. Approximate time since the first arrival of the IL-22 deficient mouse until the first *IL-22$^{-/-}$Apoe$^{-/-}$* experiment was finished: 42 months *i.e.* 3.5 years.

Regulation of IL-22

In contrast to immune cells, a limited numbers of organs have been reported to harbor cells expressing the IL-22R at steady state[210]. Most of the IL-22R expression has been localized to tissues that forms a barrier and specifically, contain epithelial cells. We observed IL-22RA1 expression in the arterial media as well as in the atherosclerotic plaque. Results from paper IV suggest that the expression level of IL-22RA1 is low in the arterial wall at steady state but tissue injury seems to increase the expression of the receptor. This is evident from our results showing that IL-22RA1 staining of injured carotid arteries are increased compared to non-injured controls.

We observed increased production of IL-22 from activated splenocytes harvested from HFD fed mice as well as a trend towards increased IL-22 levels in plasma from mice fed HFD, compared to chow fed mice. Interestingly, LDL has been reported to activate AHR in an *in vitro* model of fluid sheer stress[287]. It is therefore tempting to suggest that LDL or oxLDL can activate AHR and the subsequent IL-22 production from T cells (or other cells) during atherogenesis *in vivo*. Future studies are needed to determine which cells that produce IL-22 after HFD. Increased IL-22 levels in plasma have been reported in patients with acute pancreatitis and IL-22 is upregulated in psoriatic skin and in plasma from patients with psoriasis indicating that IL-22 is increased during inflammatory tissue responses[233,235,288]. Interestingly, it has previously been shown that IFNγ and TNFα stimulation can further upregulate IL-22RA1 expression on keratinocytes and fibroblasts, amplifying the effect of IL-22 upon inflammation[210,289]. Moreover, immune cells can release a decoy receptor inhibiting to limit IL-22 availability. The limited and what seems to be a highly controlled expression of the IL-22RA1 subunit makes IL-22 an interesting therapeutic target. In particular, targeting the receptor may be a better strategy when aiming to interfere with IL-22 biology since other cytokines related to IL-22 (IL-20, IL-24) also signals via the IL-22RA1 and can mediate IL-22-like effects in the absence of IL-22[290].

Targets of IL-22 in the vasculature

In paper IV, we suggest that medial SMCs are the main target for IL-22 in the arterial wall. This is based on IL-22RA1 stainings showing positive cells in the media of injured carotid arteries as well as in the media and in the plaque of subvalvular lesions. Isolated mouse arterial SMCs express the receptor and modulate its expression of SMC-related genes after IL-22 stimulation. We did not observe any co-localization between IL-22R staining and the SM myosin heavy chain marker in the media of injured carotid arteries, which may suggest that the IL-22R is upregulated on SMCs that down-regulate their contractile phenotype to migrate into the intima. However, other cell types in the arterial wall may also be affected by IL-22. For example, unpublished data from our lab indicates that a human microvascular endothelial cell line derived from skin express the IL-22R

and signals via STAT3 phosphorylation after IL-22 stimulation. However, staining for the receptor did not indicate positive cells at the location of the endothelium and we did not observe co-localization between the IL-22RA1 and the endothelial cell marker von Willebrand factor in the carotid artery. It remains to be elucidated if mouse and humans show the same pattern for IL-22R expression.

Interestingly, many cells in the atherosclerotic plaque seem to be positive for the IL-22RA1 subunit. This could indicate migrating SMCs but in view of the high amount of staining, other cells in the plaque may also express the receptor. Hematopoietic cells are thought not to express the IL-22R but one study report that IL-22 induces IL-1β production in human adipose tissue $CD14^+$ macrophages while the circulating precursors were not targeted by IL-22[291]. Some tissue macrophages have been reported to derive from non-hematopoietic progenitors. For example, liver Kupffer cells, microglia and pleural macrophages have been shown to proliferate and renew independently of the bone marrow[292]. In atherosclerosis, local proliferation rather than monocyte recruitment has been reported to regulate the accumulation of macrophages in the plaque in established atherosclerosis while monocyte recruitment is more important in the early stage[25]. This suggests that most of the macrophages in the vascular wall originate from hematopoietic derived monocytes. In addition, we did not observe any effect of IL-22 deficiency on macrophage or lipid content in the plaques or for the levels of macrophage related cytokines such as IL-1β, TNF-α or the monocyte chemotactic protein 1 (MCP-1) in plasma. It remains to be elucidated whether other cells in the plaque except for SMCs are targets for IL-22.

Other possible mechanisms

In paper IV, we suggest that IL-22 deficient mice develop a reduced amount of atherosclerosis because of the effects of IL-22 on SMC dedifferentiation. Hypothetically, a lower amount of medial SMCs switching from a contractile phenotype to start to migrate into the intima will result in less ECM production and hence a smaller plaque size in mice lacking IL-22.

In paper IV, we do not directly show that IL-22 stimulates migration of SMCs from the media to the intima but IL-22 has been previously reported to induce migration of pulmonary SMCs[240]. Moreover, we observed increased α-actin expression in the media while no differences was found in the plaque. Macrophages have been shown to express SMC markers and hence, discriminating these cell populations can be a challenge. This may suggest that α-actin alone cannot be used to identify dedifferentiated SMCs in the plaque and that other markers or a combination of markers should be used. We observed decreased collagen area in the plaques and conclude that this is because of the lower amount of SMCs migrating into the plaque in mice deficient in IL-22. IL-22 has previously been shown to stimulate ECM production from dermal fibroblasts and

it cannot be excluded that fibroblasts in the vascular wall also are targeted by IL-22 and contribute to the results observed in paper IV.

In our study, we used mice genetically deficient in IL-22 since birth, which means that IL-22 deficiency may have other underlying developmental or systemic effects that contribute to the observed effects on atherosclerosis. However, IL-22 deficient mice develop normally and are considered healthy[293]. According to previous publications focusing on the role of IL-22 in diabetes, lipid metabolism and defense against pathogens, other systemic mechanisms may also contribute to the results observed in our study.

IL-22 deficient mice have a compromised ability to control pathogenic infections and the homeostasis of commensals. Mice with a deficient mucosal immunity develop metabolic disorder and IL-22 has been implicated as an important mediator[242,294]. Administration of exogenous IL-22 to obese mice reduced weight gain and improved insulin resistance as well as reduced the mortality after *C. rodentium* infection[242]. Infectious agents and gut microbiota have been implicated in plaque development[295,296]. However, the role of IL-22 in alleviating metabolic disorder was not primarily in altering the gut microbiota but rather in reducing the chronic inflammation. In our *IL-22*$^{-/-}$*Apoe*$^{-/-}$ mice, we could not detect any signs of increased systemic inflammation (no difference in pro-inflammatory cytokines and even lower levels of plasma IL-6 compared to *Apoe*$^{-/-}$ mice). Chronic inflammation is believed to be one of the underlying mechanisms responsible for the accelerated development of atherosclerosis in diabetic patients[75]. However, our mice developed a reduced plaque size without signs of increased inflammation.

Interestingly, HFD fed IL-22RA1 deficient mice, but not IL-22 deficient mice, has been shown to gain more weight and develop insulin resistance[242]. This indicates that IL-20 and IL-24, which also signal through the IL-22RA1 subunit, can have compensatory effects in the absence of IL-22. In our study, IL-22 deficient *Apoe*$^{-/-}$ mice displayed increased weight compared to *Apoe*$^{-/-}$ controls while insulin or glucose levels were not measured. However, since diabetes is known to accelerate plaque development it is unlikely that these mechanisms have influenced the results in paper IV[76].

In addition, IL-22 has been implicated as an important player in the regulation of lipid metabolism. Administration of IL-22 to obese mice significantly decreased triglycerides and free-fatty-acid levels in plasma as well as preserved the liver function and promoted triglyceride lipolysis in adipose tissue[242]. We did not observe any differences in triglyceride or cholesterol levels or changes in plaque lipid deposition between knockouts and controls.

SMC growth factors and CVD in man

In paper V, we measured the levels of classical SMC growth factors (EGF, HB-EGF and PDGF) in plasma from two types of patients' i) endarterectomy patients participating in the CPIP study and ii) at baseline in healthy subjects that were followed for 15 years for incidence of CE (MDC). In the CPIP cohort, we observed a positive association between the SMC growth factor levels in plasma and collagen and elastin content of the carotid endarterectomy samples. In the MDC cohort, patients with high levels of HB-EGF had the lowest incidence of CE (Figure 8).

The growth factors in this study was measured with a PEA-technique based on two steps 1) antibodies that recognize the protein are linked to a oligonucleotide-probe which after addition of a DNA polymerase will form a DNA template and 2) the DNA template is amplified and detected using microfluidic real-time quantitative PCR. All results are expressed as arbitrary units (au), which may be a drawback of the present study. Therefore, we selected a few patients from the CPIP cohort that had high respectively low au for the SMC growth factors and analyzed the absolute concentration in these samples. Importantly, patients with high au of the SMC growth factors also had a high absolute concentration indicating that the measurements performed in this study are valid, as absolute concentrations are not needed for the association analysis presented in this paper. This also means that the major implications of paper V is to give clinical support to numerous experimental studies previously demonstrating that these SMC growth factors stimulate intimal SMCs. However, If SMC growth factors are to be used as biomarkers it is important to note that absolute concentration would be needed to be able to set up cut-off limits for risk assessment. Future studies are needed to determine if SMC growth factors may be clinically useful as biomarkers of cardiovascular risk.

We conclude that high levels of the SMC growth factors may protect by increasing the amount of SMCs in the plaques. Intriguingly, SMC growth factors correlate to elastin and collagen content in the plaques while no association was observed to SMC α-actin staining of plaque sections. It is important to note that the biochemical measurements of plaque components (MMPs, collagen, elastin, caspase 3 activity) was analyzed in plaque homogenate while the immunohistochemical stainings were performed on one section from the most stenotic part of the plaque. This was confirmed by an additional analysis performed on the endarterectomy samples.

As shown in figure 9, the percentage of SMCs differ depending on which part of the plaque that was analyzed indicating that ECM proteins measured in plaque homogenate may be a better measurement of SMC activity and plaque stability. When quantifying the SMC α-actin content at different positions in the plaque, we found a five-fold difference in SMC α-actin staining between the sections with the highest versus the lowest percentage of SMC α-actin positive area median % 17.9(9.0-24.5) vs. 3.6(1.9-5.2)). Accordingly, it is reasonable to believe that the SMC α-actin stained area of one section might not be representative for the whole plaque.

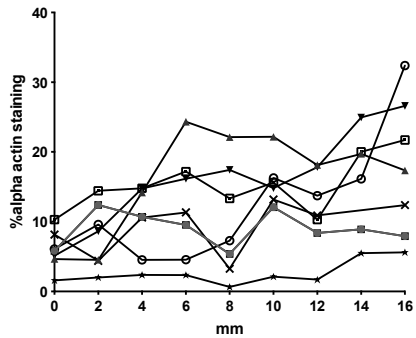

Figure 9. The graph illustrates how the percentage of SMCs (α-actin staining) varies over a plaque. Each line indicates one plaque, n=7.

Why HB-EGF?

PDGF, EGF and HB-EGF levels in plasma displayed a positive correlation with fibrous plaque content while only HB-EGF levels were associated with a reduced incidence of CE. It is interesting to note that HB-EGF show a better predictive value then PDGF and EGF because HB-EGF has not received so much attention in the field of plaque development and stabilization during the recent years. Several cell types in the plaque such as SMCs, activated macrophages and endothelial cells produce HB-EGF[297]. Except for affecting vascular SMCs, HB-EGF has been implicated in a number of physiological mechanisms such as wound healing, reproduction, angiogenesis and cancer biology. Therefore, it is difficult to know whether the levels of HB-EGF measured in plasma solely results from active ongoing processes in the vascular wall. It should be emphasized that the present study is an association study and cannot be used to prove causality and the biologically most important factor does not necessary need to be the strongest biomarker.

HB-EGF has been reported to be more potent than EGF in stimulating SMC proliferation and migration. The binding of HB-EGF, but not EGF, to cell surface heparin sulfate proteoglycans (HSPGs) has been proposed to be one explanation[55]. Although this might be considered as a weak argument, it should be noted that the

chemotactic effect of HB-EGF was reduced by almost 70%, down to the level of EGF, when the binding to HSPGs was abrogated meaning that the effect of HB-EGF interacting with HSPGs is highly efficient. HB-EGF and PDGF have been assigned the same potency in inducing SMC migration and proliferation. However, PDGF is also chemotactic for monocytes and neutrophils, which may increase the degree of plaque inflammation in addition of affecting SMC migration[298]. It might therefore be proposed that PDGF can play a dual role in plaque development and hence may not function as good as HB-EGF as a prognostic marker for CE in the MDC cohort.

Plaque size versus stability

Atherosclerotic plaques develop during decades and may for some go unnoticed throughout life. Stable plaques that rarely cause symptoms are rich in SMCs and ECM and have low levels of lipids, apoptotic cells, inflammation and a low rate of tissue degradation. SMCs are key players in regulating plaque stability but increased SMC proliferation and production of ECM can also result in plaque growth. A large plaque may compromise blood flow and certainly be unpleasant but as long as the stable phenotype remains, such a plaque rarely cause devastating symptoms such as a MI or stroke. Hence, plaque size may not be as important as plaque phenotype.

In mice, plaque size is most often used as the readout for how well a particular intervention affected atherosclerosis development. In paper IV, mice deficient in IL-22 develop smaller atherosclerotic plaques. However, there is no simple answer to whether IL-22 should be considered a "pro-atherogenic cytokine" or not. If the main role of IL-22 is to activate SMC dedifferentiation during plaque development, as the results indicate, the consequence may be a larger plaque size but possibly with a more stable phenotype. We could not detect any difference in the balance (percentage) of SMCs, lipids or macrophages in the plaques between the mouse models. Therefore, we cannot draw any conclusions whether IL-22 affects plaque stability, based solely on the data reported in this study. However, the plaques that developed in these mice were fairly small, indicating that we study the role of IL-22 in early plaque development. Because of the early time-point, it can be difficult to detect differences in markers of plaque stability, which may be more prominent later during atherogenesis. Vascular injury models that modulate the blood flow have been established to imitate the development of an unstable and a stable plaque phenotype. Future studies are warranted to study the role of IL-22 in plaque stability. Two previous clinical studies have reported increased levels of IL-22 in symptomatic compared to asymptomatic plaques as well as increased frequency of circulating Th22 cells in patients with CVD. However, it is tempting to speculate that this reflects the ongoing tissue repair

mechanisms that are initiated in these patients rather than a pro-atherogenic role of the cytokine.

A common method to assess the degree of plaque stenosis is by ultrasound measurements of IMT in the carotid artery. However, as mentioned above, plaque size may not always tell us which patients that are at risk of suffering a CE. Therefore, there is a great need for other methods or biomarkers that non-invasively can reflect the plaque phenotype and predict the risk of a future event.

Previous studies investigating the role of SMC growth factors, e.g. PDGF, in mice have reported increased neointima formation after PDGF administration while using blocking antibodies had the opposite effect[52,53]. In paper V, we observed a positive association between EGF and PDGF, but not HB-EGF, to IMT in the CPIP cohort. However, the IMT and the prognostic data are not necessarily disjunctive. We report that carotid endarterectomy patients demonstrate an association between the plasma levels of all three growth factors with the collagen and elastin contents of the plaques. This suggests that the growth factors help to stabilize the plaque by stimulating SMCs and the synthesis of ECM components. However, it is not unlikely that this response also would result in an enlargement of the plaque, which could explain the positive association observed to IMT.

Conclusions and Future Perspectives

Atherosclerosis is a chronic inflammatory disease and the underlying cause of most MI and strokes. Both innate and adaptive immune responses as well as SMC repair mechanisms have been shown to be involved in disease development making atherosclerosis a multifactorial and rather complex disease.

The first part of this thesis is focused on protective immune responses to atherosclerosis-related antigens in experimental atherosclerosis. In paper I, we developed a method to study ongoing antigen-specific immune responses to an atherosclerosis related-antigen after immunization. In particular, the matrigel method was used to study Th2 immune responses to ApoB100 but this method could also be used to evaluate other potential antigens and vaccine formulations, both in atherosclerosis and in other diseases.

ApoB100 and peptides thereof have been extensively investigated as target antigens in atherosclerosis. In particular, p210 have been successfully tested in several different immunization strategies to treat experimental atherosclerosis. Induction of mucosal tolerance using mucosal administration of p210 coupled to CTB is currently being evaluated as a potential compound to take into clinical trials. In paper II, we report that p210-CTB stimulate B cells to acquire a regulatory phenotype and induce Tregs *in vitro* indicating that B cells may be important mediators of the p210-CTB mucosal tolerance. The present study were not able to detect any evidence that transfer of p210-CTB pulsed B cells would affect atherosclerosis development but future studies are needed to fully determine if p210-CTB pulsed Bregs are important in disease development. Elucidating the mechanism behind p210-CTB in mucosal tolerance is of importance before testing the compound in patients.

Immune responses are of great importance in atherosclerosis development and indirectly also for plaque stabilization. However, one of the most central cells in plaque vulnerability and stabilization is the SMC. SMCs that proliferate, migrate and produce ECM are pivotal to preserve the vascular integrity and to prevent plaque rupture. SMCs are the main focus in the second part of my thesis.

It is well known that the immune system regulates tissue repair by for example activation of macrophages that in turn can activate SMCs. In paper IV, we demonstrate that immune cells also can regulate tissue repair by the secretion of IL-22. IL-22 was shown to stimulate dedifferentiation of medial SMCs and

possibly also their migration into the intima. This may result in a larger plaques size but more importantly, enhanced plaque stability. A lot more work is required to explore the role of IL-22 in atherosclerosis and CVD. Future work should aim to investigate the role of IL-22 in a vascular injury model to more intensively study the role of IL-22 in SMC repair responses. Moreover, IL-22 may also function as a biomarker for plaque stability in patients.

Different types of clinical assessment methods, such as ultrasound, have been developed to non-invasively study the degree of stenosis in patients. However, several factors are involved in determining which plaques are of high-risk for rupture and plaque size may not be the main determining factor for which patient that will suffer from an event. Better methods and novel biomarkers are therefore needed to detect high-risk patients.

Circulating biomarkers can be one way to predict the risk for CE. Results from paper V suggests that SMC growth factors measured in plasma can reflect plaque phenotype and in particular, high levels of HB-EGF was associated with reduced the risk for incident CE in a general population. Future studies are needed to determine if SMC growth factor are better in identifying high-risk patients than traditional risk factors and if HB-EGF could be used as a marker to predict risk of CE in the clinic. However, this study primarily highlights the importance of SMC responses in plaque stabilization.

Not only plasma proteins but also circulating immune cells have been studied as potential biomarkers. An imbalance of T effector and Treg immune responses are thought to drive atherosclerosis development. A previous report observed that high levels of Tregs at baseline protected from developing a CE. On the other hand, we demonstrate that the levels of Tregs are increased in patients with prevalent CVD compared to non-CVD controls. Also, our results show that Treg levels strongly correlate with T effector cell levels indicating that Tregs can increase to counteract the ongoing inflammatory response. Taken together, this indicates that low levels of Tregs may function as a biomarker in healthy individuals while patients with ongoing inflammatory disease may need to be considered separately. It would be interesting to study if low levels of Tregs in patients with prevalent disease could determine which patents that will suffer a second cardiovascular event. Prospective data from this particular cohort will answer this question. Overall, this suggests that the role for Tregs in CVD may be more complex than previously anticipated.

Populärvetenskaplig Sammanfattning

Hjärtkärlsjukdom är den främsta dödsorsaken i västvärlden och den bakomliggande orsaken är oftast ateroskleros. Ateroskleros, eller åderförkalkning, medför att blodkärlens väggar blir tjocka och förlorar sin normala rörlighet på grund av inlagring av fett och bildningen av ett s.k. aterosklerotiskt plack. Vissa kan leva med åderförkalkning ett helt liv utan att märka av det medan andra utvecklar allvarliga akuta symptom på grund av att ett plack ger upphov till en blodpropp som snabbt täpper igen kärlet. Beroende på vart placket sitter så orsaker detta syrebrist i hjärnan (stroke) eller i hjärtat (hjärtinfarkt).

Flertalet riskfaktorer för hjärtkärlsjukdom är identifierade t.ex. högt kolesterol, högt blodtryck och diabetes men fler metoder behövs för att upptäcka vem som kommer att drabbas av akuta symptom eller inte. I denna avhandling har jag studerat just detta samt hur ett plack uppkommer och hur man med hjälp av nya behandlingar skulle kunna förhindra dess tillväxt.

Fett som vi äter transporteras i kroppen bland annat i partiklar som kallas låg-densitet lipoprotein (LDL, det ”onda” blodfettet) och förenklat så kan man säga att ett plack bildas när fett från blodcirkulationen ansamlas i kärlväggen. LDL som lagras i kärlet kommer så småningom att modifieras på flera olika sätt, man kan säga att fettet härsknar, precis som gammalt smör. Kroppens städare, makrofagerna, kommer att kämpa för att rensa bort fettet från kärlväggen men allteftersom ansamlingen fortsätter så tappar makrofagerna kontrollen över situationen. Makrofager fulla av fett bildar s.k. ”skumceller” som till slut dör. Nivåerna av modifierat LDL tillåts nu öka ytterligare i kärlväggen vilket bidrar till att kärlet blir inflammerat. Detta resulterar i rekrytering av vita blodkroppar från blodcirkulationen och kroppen kommer att reagera precis som när vi blir infekterade av tillexempel ett virus, nämligen genom att aktivera immunförsvaret.

Immunförsvarets uppgift är delvis att försöka städa bort det okända som inte hör hemma i kroppen men i vissa fall kan immunförsvaret även känna igen och reagera på kroppens egna proteiner, t.ex. delar av modifierat LDL. En vanlig virusinfektion kommer till sist att rensas bort men vid åderförkalkning samlas mer och mer fett och immunförsvaret kommer få fortsätta att arbeta. Istället för att läka sjukdomen så förvärras utvecklingen av åderförkalkning och det bildas en kronisk inflammation i kärlväggen.

Den pågående inflammationen aktiverar även glatta muskelceller vars främsta uppgift är att producera proteiner för att försöka behålla kärlets normala struktur genom att läka den vävnadsskadan som håller på att uppkomma. Ackumulering av fett, immunceller, glatta muskelceller och strukturproteiner gör att placket växer inåt kärlet. Ett stort plack gör att blodet får det svårare att passera genom det åderförkalkade kärlet men placket kan även gå sönder vilket leder till att en blodpropp bildas. Balansen av de olika komponenterna i placket bestämmer vilka plack som kommer att orsaka akuta symptom eller inte. Mycket inflammation och låga nivåer av de läkande glatta muskelcellerna ökar risken för att placken ska gå sönder och orsaka akuta symptom. För att hitta de som har en hög risk för att utveckla hjärtkärlsjukdom så behövs det biomarkörer. Biomarkörer är något som finns i högre eller lägre nivåer hos dem som kommer bli sjuka och som lätt kan hittas med hjälp av ett enkelt blodprov.

Åderförkalkning är alltså en inflammatorisk sjukdom som kan liknas vid en virusinfektion. En virusinfektion kan förhindras genom vaccinering men tänk om man kunde skydda sig mot åderförkalkning på samma sätt? I min avhandling har jag studerat just detta, möjligheten att med ett vaccin stoppa utvecklingen av åderförkalkning. I studie I och II använder jag en musmodell för att undersöka möjligheten att skapa en skyddande immunreaktion mot åderförkalkning. I den första (studie I), vaccinerar vi möss och studerar den immunreaktion som skapas mot ett specifikt plackprotein (apolipoprotein B100, ApoB100). I den andra (studie II) så använder jag mig av celler som vi tror kan dämpa den pågående immunreaktionen i placken för att se om dessa kan stoppa utvecklingen av åderförkalkning. Ytterligare en typ av immun- dämpande cell studeras i studie III. Där har jag haft möjligheten att undersöka om nivåerna av dessa regulatoriska celler är annorlunda hos patienter med hjärtkärlsjukdom och/eller diabetes. Överraskande så visar vi att patienter med hjärtkärlsjukdom har högre nivåer av dessa celler.

I den andra delen av min avhandling så har jag studerat glatta muskelceller och mer specifikt, hur immunförsvaret kan reglera läkningen av kärlväggen under åderförkalkningsprocessen. I studie IV så har jag undersökt hur IL-22, en molekyl som utsöndras av immunceller för att kommunicera med vävnadsceller, t.ex. glatta muskelceller, påverkar åderförkalkning. Vi skapade en musmodell som saknade IL-22 och undersökte hur brist av IL-22 påverkar bildningen av aterosklerotiska plack. Vi rapporterar att avsaknad av IL-22 leder till att mössen får mindre plack men att detta även är associerat med en lägre nivå av glatta muskelceller som syntetiserar strukturproteiner. Detta leder till mindre plack men det skulle eventuellt kunna öka risken för att placken går sönder eftersom glatta muskelceller är viktiga för läkningsprocessen.

Andra molekyler som påverkar glatta muskelceller undersöks i studie V. Här mätte vi nivåerna av tre molekyler, som stimulerar tillväxt av glattamuskelceller, i blodet hos patienter och såg att höga nivåer av dessa (PDGF, EGF, HB-EGF) kunde reflektera ett stabilt plack som innehåller mycket strukturproteiner. Höga nivåer av en av dessa tillväxtfaktorer, HB-EGF, var även kopplat till minskad risk för att utveckla akuta hjärtkärlsymptom i framtiden.

Förhoppningen är att dessa studier ska bidra till kunskapen kring hur en plack bildas samt till hur vi enklare ska kunna identifiera dem som har hög risk för att drabbas av akuta symptom. I förlängningen så kan detta förhoppningsvis bidra till utvecklingen av nya terapier för att stoppa utvecklingen av hjärtkärlsjukdom.

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