Adaptive immune responses in atherosclerosis - or how to prolong the use of your yellow socks

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or

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Daniel Kolbus

ACADEMIC DISSERTATION

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If it's on paper it must be true

Adopted from H. Simpson

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List of original publications

The thesis is based on the following papers 1-5

1. Wigren M, **Bengtsson D*,** Dunér P, Olofsson K, Björkbacka H, Bengtsson E, Fredrikson GN and Nilsson J. Atheroprotective effects of Alum are associated with capture of oxidized LDL antigens and activation of regulatory T cells. *Circ Res. 2009 Jun 19;104(12):e62-70*
2. **Kolbus D**, Wigren M, Ljungcrantz I, Söderberg I, Björkbacka H, Nilsson J and Fredrikson GN. Immunization with cationized BSA inhibits progression of disease in Apobec-1/LDL receptor deficient mice with manifest atherosclerosis. *Immunobiology, 2010 Nov 19 [Epub ahead of print].*
3. **Kolbus D**, Ramos OH, Berg KE, Persson J, Wigren M, Björkbacka H, Fredrikson GN, Nilsson J. $CD8^+$ T cell activation predominate early immune responses to hypercholesterolemia in $Apoe^{-/-}$ mice. *BMC Immunol. 2010 Dec 2;11:58*
4. **Kolbus D,** Ljungcrantz I, Söderberg I, Alm R, Björkbacka H, Nilsson J and Fredrikson GN. Defective MHC class I antigen presentation in apolipoprotein E deficient mice does not influence early atherosclerosis. *Manuscript.*
5. **Kolbus D**, Andersson L, Ljungcrantz I, Hedblad B, Fredrikson GN, Björkbacka H, and Nilsson J. $CD8^+$ T cell populations are associated with carotid intima-media thickness but do not predict cardiovascular events. *Manuscript.*

* My surname was Bengtsson until September 2009.

Paper 1-3 are used with permission from the respective publisher.

List of publications not part of thesis

Wigren M, **Kolbus D**, Dunér P, Ljungcrantz I, Söderberg I, Björkbacka H, Fredrikson GN, Nilsson J. Evidence for a role of regulatory T cells in mediating the atheroprotective effect of apolipoprotein B peptide vaccine. *J Intern Med. 2010 Nov 3 [Epub ahead of print].*

Zhao M, Wigren M, Dunér P, **Kolbus D**, Olofsson KE, Björkbacka H, Nilsson J, Fredrikson GN. FcgammaRIIB inhibits the development of atherosclerosis in low-density lipoprotein receptor-deficient mice. *J Immunol. 2010 Mar 1;184(5):2253-60. Epub 2010 Jan 22.*

Daniel Kolbus*, Katarina E. Olofsson*, Maria Wigren, Jan Nilsson, Harry Björkbacka[#] and Gunilla Nordin Fredrikson[#]. High fat diet induces reduction in regulatory T cells and increased splenocyte proliferation. *Manuscript.*

*, [#] Authors contributed equally to this work

Daniel Kolbus, Maria Wigren, Irena Ljungcrantz, Ingrid Söderberg, Jan Nilsson, Gunilla Nordin Fredrikson. ApoB-100 peptide immunization do not inhibit advanced lesion progression in *ApoBec1-/-* LDLr-/- mice. *Manuscript.*

Bengtsson D, Björkbacka H. Atherosclerosis: cell biology and lipoproteins. *Curr Opin Lipidol. 2009 Aug;20(4):355-6.*

Rydgren T, **Bengtsson D**, Sandler S. Complete protection against interleukin-1beta-induced functional suppression and cytokine-mediated cytotoxicity in rat pancreatic islets in vitro using an interleukin-1 cytokine trap. *Diabetes. 2006 May;55(5):1407-12.*

Abbreviations

ApoB	Apolipoprotein B
APC	Antigen presenting cell
ApoBec1$^{-/-}$	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1 deficient mice
Apoe$^{-/-}$	Apolipoprotein E deficient mice
cBSA	Cationized bovine serum albumin
CFSE	Carboxyfluorescein succinimidyl ester
Con A	Concanavalin A
CTL	Cytotoxic T cell
CVD	Cardiovascular disease
DC	Dendritic cell
FMO	Fluorescence minus one
ELISA	Enzyme linked immunosorbent assay
FoxP3	Forkhead Box P3
HDL	High density lipoprotein
HSP	Heat shock protein
IDL	Intermediate density lipoprotein
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IMT	Intima-media thickness
LDL	Low density lipoprotein
Ldlr$^{-/-}$	LDL receptor deficient mice

LPS	Lipopolysaccaride
MHC	Major histocompatibility complex
MDA	Malondialdehyde
MI	Myocardial infarction
NK	Natural killer (cell)
NKT	Natural killer T (cell)
oxLDL	Oxidized LDL
PBS	Phosphate buffered saline
SMC	Smooth muscle cell
Tap1$^{-/-}$	Transporter associated with antigen processing 1 deficient mice
Th	T helper cell
TLR	Toll like receptor
Tr1	T regulatory cell type 1
Treg	Regulatory T cell
TNF-α	Tumor necrosis factor alpha
VLDL	Very low density lipoprotein

Introduction

How to prolong the durability of your yellow socks

In order to prohibit incidence of stroke the arteries in the neck can be opened up to remove contents of atherosclerotic lesions that may otherwise rupture. This piece of tissue is often yellowish and surprisingly solid despite the extensive tissue damage within it. Thus, the atherosclerotic lesion can be looked upon as a yellow sock that protects the rest of the body from the potentially dangerous matter within it. It is constantly eroded by detrimental processes from the inside and has to be repaired to avoid burst. As with your (yellow?) socks for foot-use the quality and thickness of the atherosclerotic sock affects durability and risk for developing severe cardiovascular events. Most of us carry yellow socks within the arteries and since they are not easy to revert a plausible solution is to stimulate construction of thicker socks with better quality. In this thesis I will present two approaches on how to reach such a phenotype. I will also elucidate the role of a specific type of immune cell that may be of importance in the deterioration of the atherosclerotic socks.

General considerations

In the following pages a brief introduction into atherosclerosis and its connections to the immune system will be presented. The overall intention of the thesis is to focus on the strengths and weaknesses of paper 1-5 by thoroughly go through the different methods used, why respective method was applied as well as discussing the impact and validity of the most important findings. For those with a prior interest in the acknowledgements section I would suggest passing by the results section to get a condensed version of the findings. I am happy to invite all others to follow the presentation of basic concepts.

Presentation of key concepts

Atherosclerosis and some theories

The word atherosclerosis is derived from the Greek *athera*, describing the gruel appearance of atherosclerotic lesions and *sclerosis* depicting the hard tissue. It was initially used in a 1904 publication by Felix Marchand claiming that atherosclerosis

was the underlying cause of obstructive processes in arteries [1]. Already in 1856 Rudolf Virchow proposed that lipid accumulation in arterial walls was caused by atherosclerosis and in 1913 Nikolai Anichkov showed that the development of atherosclerotic intima in rabbits was associated with cholesterol accumulation [1]. In 1951, a review article by Duff and McMillan [2] suggested that the accumulation of lipids in the intima of atherosclerotic lesions may be derived from the blood and together with the reports by Keys [3] on the association between cholesterol and cardiovascular disease (CVD) they founded the hypothesis that lipids are connected to atherosclerosis development. Since then there has been a growing scientific consensus on the validity of the hypothesis and in 2002 it was stated that the lipid hypothesis was "universally recognized as a law" [4]. There are however some contradictions of this "law", which is discussed in a later section. Interestingly, during the 1970s the lipid hypothesis was disregarded in favour of two other theories, the *response-to-injury* theory proposed by Ross and Glomset [5, 6] and Benditt and Benditt´s *monoclonal hypothesis of atherogenesis* [7]. The response-to-injury hypothesis argues that a continuous injury to the vascular wall will start the repair process that eventually initiates the build-up of an atherosclerotic lesion, and is still utilized today. In contrast, the monoclonal hypothesis suggested that atherogenesis was caused by a single cell clone that by continuous division assembled the lesion. It is rarely used today. An alternate theory of atherogenesis was developed by Rath and Pauling, commonly named the *unified theory of cardiovascular disease (UTCD).* It states that CVD is caused by a deficit of ascorbic acid (vitamin C) that is needed for repair of tissue [8]. Both the lipid theory and the UTCD theory agree that atherosclerotic lesions are formed due to injury of the vascular vessel wall. However, when the lipid theory states that decreasing the plasma cholesterol through diet and drug interventions inhibits disease, the UTCD theory promote increased dietary intake of ascorbic acid as the key. This thesis is mainly inspired by the response-to-injury and lipid theories, and both advantages and disadvantages of these theories will be discussed.

Development of an atherosclerotic lesion

Initial development

The morphology of an atherosclerotic lesion is initially made up of *fatty streaks* consisting of an accumulation of macrophages and stores of lipids at preferential spots in the vascular wall of great arteries. The reasons why certain areas are more prone to develop lesions are thought to depend on shear stress causing injury and increased permeability in the endothelial cell (EC) layer that lines the vascular vessel wall. High shear stress promotes EC survival while low shear stress or turbulent flow cause apoptosis, vasoconstriction, coagulation and platelet aggregation [9]. This apparent contradiction is best appreciated by noting that the ECs are evolutionary biased to

withstand high pulsative blood pressure. In contrast, low flow is a signal of danger and turbulent flow around obstructing structures, like stenoses, differs in magnitude and direction which initiates cellular stress signals. An injury to the vascular vessel wall may occur due to exposure to environmental toxins, malnutrition, infection, physical trauma from surgical intervention, hypertension or negative emotional stress. This initiates an inflammatory repair response originating from site specific cells [10] and by infiltration of circulating progenitor cells [11] leading to re-endothelization. A continuous inflammatory stimulus generates a constitutive re-endothelization which also attracts immunological cells like monocytes and lymphocytes that initiate an inflammatory vicious circle.

The role of cholesterol accumulation in injured areas is best appreciated in an evolutionary context. Cholesterol is needed for cell wall integrity and would thus be needed to repair injured tissue [12] as has been reported for lipoprotein (a), which is also accumulated in lesion prone areas [13]. This system seems to be of evolutionary advantage, since the low density lipoprotein (LDL) carrier delivering cholesterol to the tissues is more efficient than the high density lipoprotein (HDL) which transports cholesterol from the tissues to the liver for catabolism into bile acids. Apparently, such a repair system is not optimized for extensive use, since constitutive accumulation of LDL-cholesterol leads to oxidation of specific epitopes [14] and subsequent fragmentation of the LDL particle [15]. Since modified forms of LDL are considered foreign the immune system will be activated in order to remove the potentially dangerous agent. Continuous oxidation of accumulated LDL exacerbate the immune system-mediated development of the atherosclerotic lesion [14], initially by infiltrating macrophages that phagocytose oxidized LDL (oxLDL). Macrophages exert certain scavenger receptors to ingest the modified LDL which is much more efficient than normal phagocytosis but can lead to exhaustion and subsequent transformation into inert *foam cells*. This is a major (although not the single [16]) reason for development of yellowish *fatty streaks* in the vascular vessel wall. Other cells of the immune system, like T cells, Dendritic cells (DC), Natural killer (NK) cells, NKT, granulocytes and B cells contribute to the development of the chronic inflammation that leads to the development of an atherosclerotic plaque.

The advanced atherosclerotic lesion

The advanced atheromatous lesion consists of a core of cholesterol crystals covered by a soft matter consisting of dead cells, foam cells and still active immune cells. Calcifications are found in the older outer parts of the lesion whereas the relatively younger smooth muscle rich *cap* region is close to the lumen of the vessel [17]. The morphology of this part of the lesion decides whether turning into an unstable *fibro-*

lipid lesion or a stable *fibrous lesion*. The fibro-lipid lesion contains a thin fibrous cap of connective tissue that protects the inner parts of the lesion to leak into the blood vessel. Such an incident would initiate formation of a blood clot that upon rupture from the lesion site would be transported with the blood current and potentially impede blood flow in smaller arteries, thereby potentially causing myocardial infarction or stroke. The fibrous lesion constitutes a favourable morphology composed of a thicker cap region due to smooth muscle cell production of extra cellular matrix. Since the cellular turnover in the cap region is higher than in other regions [17], the clinical interventions are focused on stabilizing the cap region of advanced lesions, rather than reducing the lesion size.

Cholesterol, lipoproteins and impact on lesion development

The name cholesterol is derived from the Greek *chole*, meaning bile, and *stereos*, denoting solid. The reason for this is found in the structure and function of this molecule. It stabilizes cell membranes and is an important component in the production of bile acids. Additionally, it is part of production of fat-soluble vitamins like vitamin A, D, E and K. Cholesterol is produced with varying efficiency by all animal cells with predominance in liver and intestine.

A brief introduction to cholesterol transport

Since cholesterol is insoluble in water it needs carrier molecules for transport in the circulation. The five major groups of transport molecules are divided into chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). Briefly, these carriers differ in the ratio of lipids compared to proteins, where higher lipid content renders lower density and vice versa. The *apolipo*proteins (Apo) that encircle the lipid particles aid the lipid transport and differ in properties depending on the function of the lipoprotein particle. Chylomicrons consist of a predominance of triglycerides in comparison to phospholipids and cholesterol. It uses Apo B, C, and E for transport. The ApoB particle is present in isoforms encompassing 48% or 100% of the total ApoB molecule, were the ApoB48 form is dominating in the chylomicrons. Triglycerides are removed from the chylomicrons by a lipid enzyme called *lipoprotein lipase* (LPL) for storage in cells, which converts the chylomicrons into a VLDL particle. VLDL consists of apolipoprotein B, C and E and will continue to donate triglyceride particles to tissues and with the reduced lipid content the density increase leading to conversion to IDL. Most of the triglycerides are now donated to tissues but cholesteryl esters are still retained. IDL is composed of Apo E and ApoB making it similar to LDL which has lost ApoE. LDL consists of a single ApoB particle present in the isoform comprising 100% of the total ApoB

molecule (ApoB100). It delivers cholesterol to tissues via LDL receptors expressed on the cell surface. The size of the LDL particle has been associated to risk of developing atherosclerotic lesions [18], possibly due to increased adherence of small particles to the endothelium, where they can be modified and targeted by the immune system. Since the LDL particle consists of a single ApoB100 copy, a quantification of the number of ApoB100 copies corresponds to the number of LDL particles. Subsequently, were LDL count may bias large LDL particles, the ApoB100 count quantifies the total amount of LDL particles, which may be a more valid measurement. The HDL particle, which is the smallest apolipoprotein particle, is composed of ApoA and ApoE particles. It transports cholesterol from the tissues to the liver or steroidogenic organs for conversion into bile acids and removal via the bile or recirculation through the intestine. If transported to adrenals, ovaries or testes the cholesterol is used in the production of steroid hormones.

An imbalance in the apolipoprotein transport system may occur due to reduced clearance via LDL receptor and ApoE dependent pathways. ApoB100 containing particles are able to bind to LDL receptors and ApoB48 particles are re-circulated via uptake of ApoE. Since ApoB100 is predominant on LDL particles and ApoB48 dominates larger lipoproteins, targeted inhibition of respective pathway give different outcome. Thus, mice that lack LDL receptors accumulate LDL particles, while *Apoe*$^{-/-}$ mice have higher levels of larger lipoprotein particles, like chylomicrons [19]. Subsequently, disruption of different pathways lead to imbalance of the lipoprotein transport system that may initiate atherogenesis.

Impact of dietary cholesterol on cardiovascular disease

The absorption of cholesterol occurs via the small intestine, where about one third is exogenous cholesterol from diet and the majority from endogenous sources, like the bile and the intestinal epithelium [20]. Consequently, the results from clinical studies indicate that dietary cholesterol source constitute a limited risk for CVD and atherosclerosis but that there are large variations in genetical predisposition [20]. For instance, a slight increase in dietary cholesterol reduced intestinal cholesterol absorption in one study [21], whereas the cholesterol absorption was increased in a similar study, but with extreme variation [22]. This, together with the report that just about 50% of the patients with manifest CVD are hyperlipidemic suggests that other risk factors contribute [23]. Indeed, although dietary fat and cholesterol intake is directly correlated to CVD in simple correlation analyses [24-26], this correlation is less evident in analyses taking into account several risk factors of CVD [27, 28].

Overview of the immune system

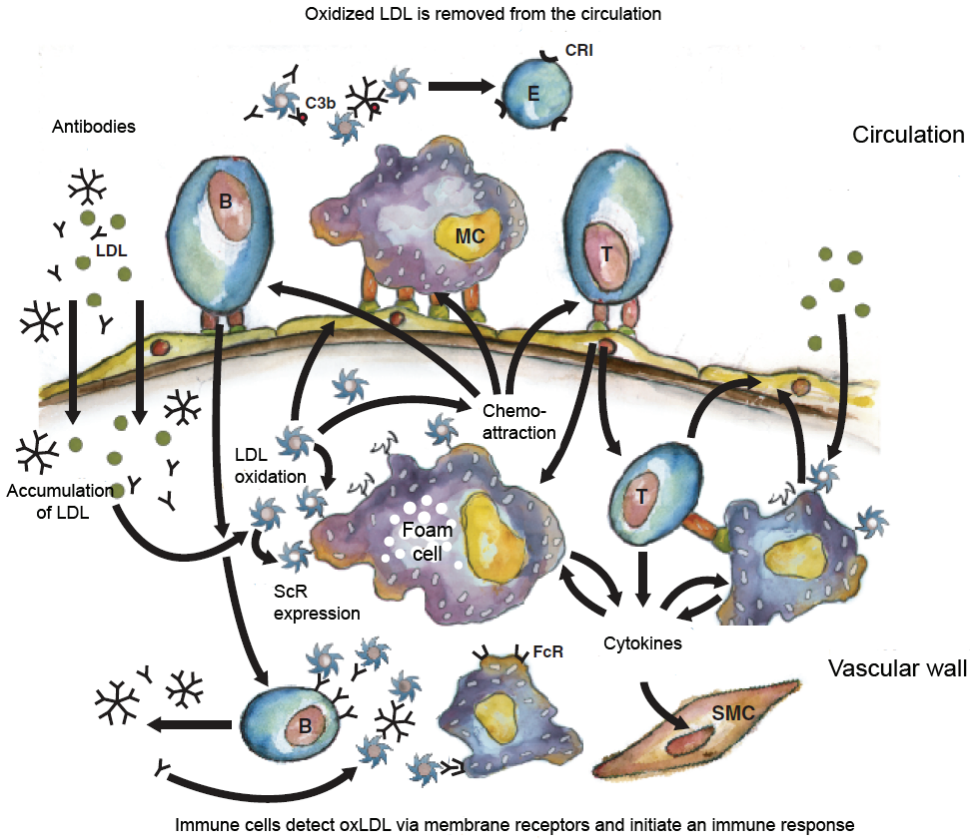
The vertebrate immune system is typically divided into two parts, based on the *broad-spectrum* efficiency in mounting an inflammatory response upon the encounter of a pathogen and the *specific* efficiency in which certain epitopes on a pathogen are targeted.

Innate immunity in the atherosclerotic lesion

The *innate* immunity consists of cell types with *pattern recognition receptors* (PRR) that respond immediately to certain *pathogen-associated molecular patterns* (PAMPs), like bacterial DNA, double stranded RNA and lipopolysaccaride (LPS) which is considered non-self. Such activation can also be targeting modified self antigens, like oxLDL. Scavenger receptors constitute one form of PRRs. It is present on macrophages to facilitate phagocytosis of foreign material like modified LDL particles. This is a potential mechanism of cholesterol removal meditated by the ABC-A1 efflux pathway. In this *reverse cholesterol transport way,* cholesterol is loaded onto Apolipoprotein-A1 and Apolipoprotein E in lipid poor HDL particles that remove cholesterol from the tissue. However, due to a malfunctional negative feedback system, intake of cholesterol via scavenger receptors can lead to pathological cholesterol levels in the cell, and subsequent cell death [29].

Another form of PRRs is the Toll-like receptors (TLR), originally reported as "Toll" receptors being important for immune system function in the fruit fly *Drosophila melanogaster* [30]. TLRs act as signalling receptors that quickly initiate an immune reaction in response to detection of a pathogen [31]. Additionally, TLRs, together with other PRRs, like CD36, natural antibodies and C-reactive protein recognize host-derived oxidation-specific epitopes denoted "danger- (or damage) associated molecular patterns" (DAMPs) [33]. Indeed, they constitute a first line of defence against potentially detrimental processes induced by modification of self-proteins. However, absence of TLRs is associated with reduced development of atherosclerotic lesions in hypercholesterolemic *Apoe*$^{-/-}$ mice [32], suggesting a pro-atherogenic role in response to the exacerbated inflammatory environment in the developing lesion.

Apart from macrophages, monocytes, DCs, B1 cells, NK cells, mast cells and the complement system is considered to be a part of the innate immune system.



Overview of the oxLDL-related immune system responses that initiate lesion development. Oxidized LDL is phagocytosed by macrophages in the vascular wall that eventually lead to transformation into foam cells. Macrophages and other antigen presenting cells induce a T- , B- and smooth muscle (SMC) cell response by direct cell contact and cytokine secretion. This leads to recruitment of more monocytes (MC), B- and T cells from the circulation or adventitia as well as SMC migration from the tunica media to the intima. B cells activated by oxLDL produce antibodies that mark oxLDL particles for phagocytosis or stimulate effector cells via Fc-receptor binding (FcR). Additionally, circulating antibody-coated oxLDL is removed by the complement system (via C3b) and erythrocytes (E; via Complement Receptor 1).

Adaptive immunity and the connection to lesions

Development of the adaptive immune system is thought to have occurred approximately 500 million years ago in jawed fish. The human adaptive immune system is defined by presence of lymphocytes with T- and B cell receptors, gene rearrangement via the recombination activating gene (RAG), Major histocompatibility complex (MHC) I and II, somatic hypermutation, and specialized primary and secondary lymph nodes [34]. Essentially, it leads to an extensive array of diversity in pathogen epitopes that can be recognized continuously throughout life. The emergence of such an immune system was rapid and has been described as a "Big Bang" [35], probably rendering evolutionary advantage to the jawed vertebrates.

Cell mediated responses

Antigen presenting cells (APC) continuously scout their surroundings for potentially harmful antigens. The antigens are presented via MHC molecules on the surface of APCs to T cells. The conformity of the antigen and T cell receptor together with the stimulatory response from the APC decides the mode of activation to be absent, pro- or anti-inflammatory. Dendritic cells are the most efficient APCs and occur predominantly in peripheral tissues. Upon recognition of a potentially harmful antigen they migrate to draining lymph nodes for antigen presentation to T cells. Antigen presentation via the MHC class I molecules activates $CD8^+$ T cells while $CD4^+$ T cells are activated upon presentation via MHC class II molecules. All cells in the body continuously express MHC class I molecules complexed to intracellular antigens. This is a "don't kill me" signal to $CD8^+$ T cells that explore signs of pathogen antigens in infected cells and of tumour cells. Presentation of extracellular antigens usually occur via the MHC class II pathway, although $CD8^+$ T cells can be activated via *cross presentation* [36] of exogenous antigens on MHC class I molecules. DCs also present lipid antigens to natural killer T cells (NKT) via the CD1 receptor. Both $CD4^+$ and $CD8^+$ T cells are present in the atherosclerotic lesions and has been shown to react to modified human plaque antigens [37-39]. Although $CD4^+$ T cells have been shown to exert pro-inflammatory effects [40], regulatory $CD4^+$ T cells are anti-inflammatory which reduce the development of the lesions [41, 42]. The role of $CD8^+$ T cells is less characterized and will be elucidated more in paper 3-5 of this thesis.

Humoral immune responses

Antibodies specific to pathogen antigens are produced by B cells and function by coating the target which stimulates phagocytosis by macrophages and other phagocytes. B cell activation occurs dependently or independently of T cell

stimulation. Reports from T cell deficient mice show that B cell antibody production of IgM isotype alone yields an efficient response to pathogens. The response is initiated by cross-linking of surface bound IgM antibodies that recognize common pathogen epitopes and is therefore considered to be an innate immune response. However, $CD4^+$ T cells can efficiently induce activation of B cells and thereby initiate antibody class switching from IgM to production of IgG, IgA or IgE antibodies. The activated B cell transform into a plasma cell that produces antibodies being secreted rather than membrane bound. Such a mature B cell reaction initiates a defined immune response depending on antibody isotype. Plasma antibody concentration is often used as a risk marker of several autoimmune diseases, but may also be part of a protective response. Human plasma contains antibodies directed towards modified LDL epitopes which are correlated to the extent of cardiovascular disease [43]. Immunization with native or modified peptide sequences corresponding to these epitopes induced an anti-atherogenic response characterized by increased IgG and IgM antibody levels, as well as reduced lesion development in mice [44]. Immunization with other epitopes of modified LDL exert similar effects [45], indicating that antibody responses has an important role in regulating atherosclerosis development. In mouse, the presence of IgG antibodies of the IgG1 subtype is correlated to anti-atherogenic effects whereas IgG2a antibodies are associated to pro-atherogenic effects. *Apoe*$^{-/-}$ mice on a C57BL/6 background with increased IgG2a (actually IgG2c since IgG2a is not expressed in this particular mouse type) develop larger lesions than mice with predominance of IgG1 antibodies towards modified LDL [46]. In humans, IgG1 is associated with pro-inflammatory responses while IgG4 is considered anti-inflammatory. Intriguingly, injection of human IgG1 antibodies specific for a modified peptide sequence of ApoB-100 reduced the amount of atherosclerosis in mice [47]. According to these results, the isotype of antibodies targeting modified LDL antigens is not determining the atherogenicity *per se*. However, one has to remember that this approach contained mixing of human and mouse antibodies, and that several reports have stated a species specific association between isotype and atherogenicity.

Provoking the immune system - immunization strategies

Immunization is a way of inducing an immunological response against an antigen by pre-exposure to the antigen. Repeated injection of an antigen starts a rapid response from specific memory cells of the immune system which is stronger than the initial response to the antigen. This is used in vaccination towards virus disease in order to commence an inflammatory response towards invading viruses. Usually an adjuvant is used to give an additional stimulation of the immune response. There are a few different adjuvants for human use, of which *Alum* is the most used. Additional adjuvants exist for use on experimental animals, but are not permitted for human use

due to safety concerns. The immunization strategy of paper 1 and 2 in this thesis is based on initiating immunologic tolerance towards modified self proteins. This originates from the finding that immunization with LDL or oxidized LDL reduced atherosclerotic lesions in rabbits [48]. This has been repeated in mouse immunizations using modified LDL particles or homogenate from lesions [49]. Additionally, immunization with specific peptides present in the ApoB-100 particle coupled to a carrier molecule (cationized BSA) and with alum as adjuvant decreased atherosclerotic lesion size [44]. In paper 1 we showed that immunization with alum alone generates an anti-atherogenic immune response, suggesting that alum is an appropriate adjuvant for immunization studies were the goal is to initiate an anti-inflammatory immune response. In paper 2 we developed this concept to also elucidate the impact of the peptide carrier molecule cBSA and showed that this can initiate an atheroprotective immune response.

Aims of the thesis

To asses the anti-atherogenic effects of alum and cationized BSA upon immunization

To characterize the immune response to early hypercholesterolemia

- locally in lymph nodes in close conjunction to the aortic tree and at distant sites
- systemically in blood and spleen

To elucidate the pro-atherogenic effect of $CD8^+$ T cells in hypercholesterolemia

To asses the association between $CD8^+$ T cells and cardiovascular end-points in humans

Ethical perspective

The most obvious ethical dilemma I faced in preparing the papers of this thesis is how to approach the fact that a lot of mice were killed to produce results that not necessarily will be of gain for humans or mice. As a consequence of this I will discuss the following questions;

I. Where there valid alternatives?

II. Where there unnecessary steps in the experimental handling?

Mice

I. Atherosclerosis is a multifactorial disease that develops over a long period of time even in mice. Thus, a full model for this disease has to monitor all the shifts in balance of for instance metabolic and immunological factors during disease development. Since we do not know all the complex relationships that shape the disease, it is apparently a difficult task to construct a non-animal system in which to test whole system hypotheses. Even if one decides to use an animal system to test an intervention, the probability of finding a true relationship is pretty low, due to the massive amount of unknown parameters that could affect the outcome [50]. In contrast, if the hypothesis is based on a separate part of the disease process involving intervention in a certain cell type, there is less need for use of animal models. However, if the overall scientific goal is to understand the *in vivo* interplay between cell types, this approach may be of less validity. The aim of the research in the Experimental Cardiovascular Research Unit is to understand the role of the immune system in atherosclerosis in order to develop vaccines. Therefore, the most valid approach is to study interventions in animal models and use cell based systems as a complement to study findings in the animal model in detail. Using only computer assisted simulation or pure cell based systems would probably generate a larger amount of misconceptions. Such research would not gain the overall aim of the research group and is therefore unnecessary.

II. Due to economical, spacious and experimental concerns it is not possible to study mice in their natural environment. Consequently, breeding several mice in the same cage is to overstep some of their natural habits and behaviour. It is however less obvious that such a handling give rise to unnecessary stress, since the natural environment as such is a stressful environment. Potentially unnecessary treatment of the mice in the papers 1-4 contained

a. shift from chow diet to high fat diet
b. injection of alum-(carrier) compound
c. blood sampling via the saphenous vein
d. euthanization

Both the diet shift and euthanization is necessary to analyze the experiment. The injection volume of alum conjugations is rather large compared to the size of the animal and could have been titrated for smaller volumes in order to prevent potential animal suffering. Contrary, the cutaneous/sub-cutaneous space of mice is more stretchable than in humans indicating that the mouse pain perception of such an intervention may be less than in humans. However, reducing the amount of immunizations in paper 2 may have resulted in a similar outcome and could have been thoroughly titrated. On the other hand, such a titration process would have required more mice, raising the question of the necessity of the titration process. Out of points a-d, *c* could have been suspended due to the little aid in understanding of the atherosclerosis process in comparison to the thought suffering of the mice. This procedure included blood sampling from conscious mice, which may have experienced considerate stress during the procedure and discomfort after the procedure. Further, such an intervention may distort the immune response possibly influencing the effect of other interventions on the atherosclerosis development. Thus, analysis of the blood sampling in paper 2 could have been performed on splenocytes harvested at euthanization and the blood sampling in paper 3 and 4 could have been suspended since it did not add essential information.

Fetal calf serum

Another less discussed ethical question with no consideration in the local ethical committee is the use of fetal calf serum (FCS). This is an essential component of almost all cell culturing used since mouse serum is too expensive. It is estimated that around half a million litres of FCS is produced worldwide every year, which essentially means that around one million bovine fetuses are killed every year [51]. A typical serum collection includes slaughter of a pregnant cow and removal of the uterus upon removal of the cow's internal organs. The fetus is removed from the

uterus, a needle is inserted between the ribs into the heart and the blood pumped out by a vacuum pump system. This procedure takes 2-5 minutes and the period from slaughter of the mother to end stage FCS collection is between 10-35 minutes, depending on staff [51]. The fetus may experience considerate pain upon heart punction (usually non-anesthetized) but it has been reported that the fetus dies within minutes after slaughter of the mother [52], and consequently may not be conscious during the blood collection.

In 2006 the International Serum Industry Association (ISIA) was formed which have the goal of setting up standards for the serum producers world wide, not only to highlight ethical concerns but also to improve traceability and safe use of serum products. We use FBS Gold from PAA, which is a member of ISIA and according to the company Serum Brochure [53] "skilled operators remove the blood from the foetus". It does not say if special ethical concerns are undertaken in this process.

I and II. Taken together, it cannot be ruled out that both mother and fetus feel considerate pain during this process. The slaughter procedure may have improved due to serum quality concerns from the industry, which may have resulted in fewer events of animal cruelty. From an ethical point of view, I still find the concerns in reducing potential animal suffering inadequate. An alternative to serum based media is serum free media (SFM) that contains unknown supplements instead of serum. It is a potential alternative, although one has to evaluate the effects in the typically used assays thoroughly. I have used SFM (Invitrogen) in a pilot experiment were it generated slightly diverging results compared to the serum based media, highlighting the need for cautiousness if changing system.

Finally, there are probably other reagents used in a typical research lab that directly or indirectly cause pain to animals or destruction of the environment. In all these potential violations of life and welfare of other organisms one has to consider if the use of these goods is necessary. I have made the decision to perform the laboratory experiments presented in this thesis bearing in mind possible detrimental effects.

Methods

Mouse models and mouse diets

Why mice – reasons for using mice in atherosclerosis research

The usage of mice in research offer a number of advantages compared to other animal models and human tissue. First, the use of inbred strains offer genetic homogeneity which limit the number of confounding factors and decrease the need for large cohorts in order to detect response to an intervention. Second, the large number of genetically modified mouse strains offers numerous possibilities in studying the effect of single genes. As an example, by 2004 more than 5000 mouse genes had been knocked out [54]. Additionally, inter-breeding of different mouse strains generate new strains tailored for a specific hypothesis. Third, the small size and relatively rapid generation of mice is an advantage compared to other animal models typically used in research.

Hazards in using mice as a model system for human atherosclerosis

Although genetic comparisons of the mouse and human genome resulted in species specific differences in only about 300 genes [55] there are strict variations on the morphological and functional level. First of all, mice are apparently much smaller than humans, which naturally also mean that the mouse organs are smaller and contain fewer cells. As a consequence, the number of cells and tissue quantities that can be isolated is lower in mice compared to humans. Although the vast majority of organs are related and positioned at similar spots, there are a number of important functional differences affecting atherosclerosis development. The lipid composition of mice is rather different than in humans, with larger portion of HDL particles compared to VLDL and LDL particles [56]. Additionally, mice do not have cholesterylester transfer protein (CETP) activity, which in humans have been linked to increased susceptibility of atherosclerosis [57]. Furthermore, due to the function of the ApoBEC-1 enzyme, mice exert an editing capacity of the ApoB protein resulting in ApoB particle length of both 48% and 100% of the molecule. The presence of the truncated ApoB-48 form likely leads to increased ApoE binding and subsequent clearance from plasma [58]. Together with previous points this clearly makes the mouse model less useful as a predictor of human atherosclerosis. Here the rabbit

model has an advantage since composition of lipoproteins containing Apolipoprotein B is similar to humans. Moreover, as in humans but unlike mice rabbits have no ApoBEC-1 enzyme editing activity, generating full length ApoB-100 lipoproteins and have functional CETP activity resulting in a profound susceptibility of atherosclerosis [59]. On the immunological level the overall composition is rather similar between mice and humans. However, the balance between neutrophils and lymphocytes varies. Mouse blood contains a higher fraction of lymphocytes constituting 75-90% compared to 10-25% neutrophils. In contrast, human blood consists of 30-50% lymphocytes and 50-70% neutrophils [60, 61]. Whether this has any functional role is not fully appreciated but will be discussed more in a later section. Altogether it may be a good idea to always cautiously evaluate mice to men comparisons to avoid developing misconceptions.

Mouse models used in the papers of this thesis

C57BL/6

The C57BL/6 mouse strain is typically used as a control strain when performing studies using genetically modified mouse strains. Among the most common mouse strains like C3H and Balb/c, the C57BL/6 strain is more susceptible to develop atherosclerotic lesions, which mostly appear in the aortic root [62]. The lesions are however usually small and even after a prolonged intake of a diet enriched in saturated fat and cholesterol the lesions are concentrated at the aortic root [62]. Most genetically modified mouse strains used for research on atherosclerosis are bred on a C57BL/6 background which is more susceptible to atherosclerosis development than other strains [63, 64]. Interestingly, female mice of C57BL/6 background develop larger lesions compared to male counterparts [65], which is opposite from the situation in man [66]. The reduced susceptibility to atherosclerosis in C57BL/6 mice in part derives from the absence of the plasma protein CETP, responsible of cholesteryl ester and triglyceride transport between lipoproteins. CETP has been linked to increased risk for coronary heart disease in human and lower serum levels of CETP is associated with longevity [57, 67]. In our laboratory we use C57BL/6 mice as control to gene targeted mice when monitoring the effect of an intervention on the lipid levels, atherosclerosis and the immune system. They usually have plasma cholesterol levels below 100 mg/dl on a chow diet and below 150 mg/dl if given high fat diet.

The C57BL/6 mice (originate from Taconic and the Jackson Laboratory) was used as a normo-cholesterolemic control model for the hypercholesterolemic *Apoe*$^{-/-}$ mouse model used in paper 1 and 4.

Apoe$^{-/-}$

Apolipoprotein E (apoE) is predominantly produced in the liver and brain [68] and commonly present on lipoproteins like chylomicrons and HDL but not LDL particles [68]. It interacts with receptors for lipoprotein removal, for example the LDL receptor, and removal of ApoE constitutes a higher prevalence for atherosclerotic lesion development even in a system unprovoked by high fat diet [58, 69]. Mouse apolipoprotein B particles exist in a full-length form which is cleared from plasma via the LDL receptor and as a partial form (48% of the full-length form) which is dependent on ApoE particles for plasma clearance via the LDL receptor related protein [70]. The deficiency of ApoE mediated clearance in *Apoe*$^{-/-}$ mice results in elevated plasma ApoB-48 in mice receiving chow diet [19]. Additionally, mice deficient in ApoE have at least five times higher plasma cholesterol levels compared to *Apoe*$^{+/+}$ mice, largely composed of very low- and intermediate density lipoprotein particles (VLDL and IDL) but also low density lipoprotein (LDL) particles [71, 72]. Concomitantly, these mice develop extensive lesions primarily in the aortic arch and to a lesser extent in descending aorta, which is augmented by a high fat diet. Initially, the lesions contain a large number of foam cells but gradually develop into more complex lesions with accumulation of cell debris and cholesterol surrounded by a fibrous cap [73]. *Apoe*$^{-/-}$ mice given chow diet develop extensive lesions after 35 weeks of age and this process is further accelerated in mice given high fat diet [73]. Although the *Apoe*$^{-/-}$ mouse model is commonly used in atherosclerosis research one has to remember that the ApoE molecule has heterogeneous effects on its surroundings. A non-metabolic property is the anti-inflammatory function of ApoE on lymphocytes reported already in 1982 by Avila and coworkers [74]. Since then, Apo E function has been associated with a decreased type 1 inflammation by modulation of IL-12 production [75]. Additionally it suppresses neutrophil activation [76] but can also act as a scavenger of exogenous lipids to activate T cells [77]. As ApoE has several functions the inhibition of ApoE expression may affect an array of processes, including modulation of inflammation [78]. Thus, it is not a pure model of malfunctional lipid metabolism and the interpretation of immune system interventions have to be performed with cautiousness.

The *Apoe*$^{-/-}$ mouse model (Taconic and the Jackson laboratory) used in paper 1, 3 and 4 provided a hypercholesterolemic environment to test diet- or immunization interventions.

ApoBec-1 / LDL receptor deficient

The use of mice to model human disease is limited in *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice due to differences in the ApoB particles. While the ApoB particles in human liver typically consists of a full-length form (ApoB100) the mouse ApoB is 48% (ApoB48) of the

full-length form due to the Apolipoprotein B messenger RNA editing enzyme catalytic polypeptide-1 (ApoBec-1) enzyme activity [79]. In order to adjust the mouse lipid profile towards the human profile, Powell-Braxton *et al.* constructed a LDL receptor deficient mouse model with malfunctional ApoBec-1 activity [80]. The resulting *Ldlr*$^{-/-}$*Apobec1*$^{-/-}$ mice had elevated plasma LDL and ApoB100 levels similar to the lipid profile in humans with familiar hypercholesterolemia [80]. Moreover, compared to *Ldlr*$^{-/-}$ mice or *Apobec1*$^{-/-}$ mice they developed extensive lesions after 8 months on chow diet or 8 weeks on high fat diet. Since *Ldlr*$^{+/+}$*Apobec1*$^{-/-}$ mice on a C57BL/6 background have normal plasma cholesterol levels, the combined effect of targeting the LDL receptor and ApoBec-1 generates elevated cholesterol levels and atherosclerotic lesions. The lesion morphology in *Ldlr*$^{-/-}$*Apobec1*$^{-/-}$ mice ranged from foam cell enriched fatty streaks to advanced plaques with smooth muscle cell infiltration and fibrosis of the intima [80].

The ApoBec-1 / LDL receptor deficient mouse model used in paper 2 (C57BL/6 129S-*Apob*^{tm2Sgy} *Ldlr*^{tm1Her}/J originates from the Jackson Laboratory) has a mutation in the ApoB48-editing codon of the mouse ApoB gene which inhibits cleavage into the ApoB48 form by the ApoBec-1 enzyme. This provided a human-like model to elucidate the response to an alum-cBSA immunization.

Tap1$^{-/-}$ *and Apoe*$^{-/-}$*Tap1*$^{-/-}$

Most cells continuously express endogenous peptides via MHC class I molecules in order to alert connecting CD8^{+} T cells that their system is tolerable or that the system is malfunctional due to a virus attack or cellular damage. The Transporter associated with Antigen Processing 1 (TAP-1) is crucial for transport of peptides from the cytoplasm into the endoplasmatic reticulum (ER) where they can bind to MHC class I molecules [81]. Mice deficient in TAP-1 have reduced levels of surface bound MHC class I molecules as well as a reduced CD8^{+} T cell population [82]. According to paper 4, the plasma cholesterol and triglyceride levels were normal compared to C57BL/6 mice. Although the mice had partly bold areas in the back after long time high fat diet treatment we and others did not see adverse effects in life expectancy of this mouse model (study 4 and [82]).

We generated an *Apoe*$^{-/-}$*Tap1*$^{-/-}$ mouse model by crossing *Apoe*$^{-/-}$ mice (C57BL/6 background, Jackson Laboratories) with *Tap1*$^{-/-}$ mice (C57BL/6 background, Jackson Laboratories). The offspring followed Mendelian inheritance generating mice homo- and heterozygous for *Apoe* and *Tap1*. Preferably the homozygous offspring were allowed to breed, increasing the probability of homozygous *Apoe*$^{-/-}$*Tap1*$^{-/-}$ offspring.

The *Apoe*$^{-/-}$*Tap1*$^{-/-}$ mice had plasma cholesterol and triglyceride levels comparable to *Apoe*$^{-/-}$ mice. This mouse model did not develop bold areas in the back as the *Tap1*$^{-/-}$ mouse model and appeared healthy.

The *Tap1*$^{-/-}$ and *Apoe*$^{-/-}$*Tap1*$^{-/-}$ mouse models used in paper 4 (originating from the Jackson Laboratories) made it possible to study TAP1 independent immune responses to a hyperlipidemic environment.

Mouse diets – from marshmallows to synthetic diet

Wild mice usually consume a low caloric diet and to mimic this diet in the laboratory milieu the chow diet was developed consisting of approximately 4-6% vegetable oil fat and <0.02% cholesterol [56]. This diet is composed of a variety of vegetable products like wheat, corn, oats or soy bean and protein content from fish [83] and is lower in fat compared to most human diets in developed countries [71]. Since the most used inbred mouse strains are less susceptible to spontaneous atherosclerosis a diet high in fat and cholesterol is normally used to initiate lesion development. The types of high fat diets are numerous, from the "supermarket diet", allowing experimental rats free access to a variety of snack foods like marshmallows, salami, cheese and peanut butter [84] to the synthetic diets of today. Already in the 1950s, Wissler *et al.* composed the first atheromatous diet which contained bile salts or bile acid to stimulate atherosclerosis in rats [85, 86]. In comparison, a diet consisting of 30% cocoa butter, 5% cholesterol and 2% sodium cholate induced myocardial infarction in rats [87]. This diet was further modified to elucidate factors that would increase the number of infarcted animals. Despite using 775 rats divided into 49 groups receiving 38 different kinds of diet, the authors could not find a more infarction-prone diet. However, they concluded that omission of propylthiouracil, sodium cholate, cholesterol or/and fat lowered the infarct incidence [88]. This "Thomas-Hartroft" diet was used in a mix 1:3 with chow diet to produce the *Paigen* diet, consisting of 15% fat, 1.25% cholesterol and 0.5% cholic acid [89]. Female C57BL/6J mice receiving this diet developed lesions in heart and aorta after 14 weeks of diet and extensive (~8 %) lesions after 9 months which was independent of seasonal changes [89].

Atherogenic diets often lead to liver damage and gallstone formation in the mice. Therefore, Nishina and coworkers tested synthetic low- and high fat diets with well-defined composition in order to produce aortic lesions without confounding factors coupled to unknown materials in the diet. They concluded that a diet consisting of 50% sucrose, 15% cocoa butter, 1% cholesterol and 0.5% sodium cholate induced a

decrease in plasma HDL and the highest increase in VLDL and LDL with a concomitant development of aortic lesions without inducing gallstone or liver damage [90]. Interestingly, a high fat diet consisting of coconut oil induces lower plasma cholesterol levels than corresponding diets consisting of cocoa butter. This is probably attributable to the difference in fatty acid composition where the long chain cocoa butter induces more production of liver cholesterol than the coconut oil [90]. The impact of the source of fat for atherosclerosis development was further elucidated by Nishina *et al.* including both plant and animal fats in the analysis. Using the same diet composition of non fats as in reference [90], C57BL/6 mice were given a diet containing either hydrogenated soy-, coconut- or palm oil as well as cocoa- or dairy butter, pork- or beef fat. Thus, the diets varied the content of mono/polyunsaturated fats and saturated fats of long or short chain composition. With the exception of coconut oil, mice that received saturated fats developed largest lesions in the aorta. Dairy fat was inducing largest lesions but in none of the diets lesion size was correlated to plasma cholesterol [91]. According to the findings by Thomas and Hartroft [88] also the cholesterol content affected atherosclerosis and myocardial infarction. To optimize the quantity of dietary cholesterol that induced most atherosclerotic lesions, Nishina *et al.* analyzed cholesterol contents of 0.5% and 1.0% of total diet in C57BL/6 mice. Although none of these amounts generated any difference in lesion size or plasma lipids compared to control diet, the lesions were more profound in the group receiving 1% cholesterol [90]. In these experiments, a cholate (cholic acid) was used in an optimized concentration of 0.5% of total diet. Due to its heterogeneous effects on lipid metabolism, cholate is rarely used in studies of today. Initially it was included in atherogenous diets because of drastic effects on atherosclerosis development. It has however several effects on lipid metabolism and inflammation that increase complexity in analysing the impact of diet and other interventions in the animal model of choice. For instance, cholate induces hepatic genes involved in liver fibrosis [92] and it was recently reported to induce giant cell formation in atherosclerotic plaques of *Apoe*$^{-/-}$ mice receiving Paigen diet. This was associated with increased Cathepsin K activity leading to elastin degradation and smooth muscle cell migration [93]. The same group also argued that a high fat diet including cholate induced lung inflammation similar to human sarcoidosis [94]. Although interesting for studies of these particular syndromes cholate clearly disturbs immune cell responses and thus may distort pro- or anti-inflammatory interventions.

The most used high fat diet today contains 21% milk fat and 0.15% cholesterol [56]. It was originally used by Plump *et al.* [71] in the publication of the *Apoe*$^{-/-}$ mouse model were it provided a rapid increase in lesion development. The atherogenicity of the cholesterol content in the diet was adjusted in a study with *Ldlr*$^{-/-}$ mice receiving a low fat diet with a cholesterol content of 0%, 0.02%, 0.15%, 0.3% or 0.5%. The three higher concentrations induced similar plasma cholesterol levels and lesion

development [95], suggesting that the use of 0.15% cholesterol is close to the critical point of developing disease. Moreover, it also highlights the atherogenic properties of cholesterol irrespective of the fat content in diets given to *Ldlr*$^{-/-}$ mice.

The high fat diet used in our laboratory is composed of 21% fat from cocoa butter, 0.15% cholesterol and 17.2% protein. Cocoa butter does not contain cholesterol, in comparison to milk fat which usually contains 0.05% cholesterol and thus add up to a total cholesterol content of 0.2% in these high fat diets [56]. As milk butter, cocoa butter consists of saturated fats and is commonly used by other research groups. The diet induce an increase in atherosclerosis but the lesion size in aortic arch and aorta is usually less than in reports by others. We do not think however that the diet is the major factor explaining the deviation. Instead, the environment in the animal facility may affect the extent of atherosclerosis. The current animal house is lower in pathogen frequency compared to many other animal facilities, which may induce less pathogen stress in the mice. This is more thoroughly elucidated in a later section. Finally, when comparing animal groups receiving chow diet with animals receiving high fat diet, one has to keep in mind that while the constituents of the high fat diets are reasonably standardized the nutrient composition of the grains in the chow diet may vary with season, which could induce deviations in the "healthy control group" [83]. The chow diet in our lab consists largely of barley and wheat with a 5% fat content and 21% protein content. Both the chow- and high fat diet is produced by Svenska Lantmännen.

Immunization – reagents and strategy

Aluminium adjuvants, "Alum"

History

Aluminium containing adjuvants has been used in human vaccines for more than 60 years and are considered a safe and effective way of generating Th2 associated immune responses [96]. Alum is a class of chemical compounds of different origin but sharing the empirical formula $AB(SO_4)_2*12H_2O$. It has a wide range of uses ranging from a flame retardant in clothing, in aftershave, waxes for removing body hair, preservative for fruit and vegetable crispiness, play-clays for children and purification of drinking water in industries. Interestingly, alum was used by bakers in England during the 19th century to make the bread whiter. The whiteness of the bread was considered a sign of quality in the milling process which differentiated the wealthy from the poor eating brown bread. Evidently, alum was used to mask bad grains and milling which made the Church of England criticise the whole idea of white bread as beneficial for the English society and especially the use of alum in

baking [97]. Later, the "Sale of Food and Drugs Act" banned alum as an ingredient in bread baking.

The use of alum adjuvants in medicine was originally tested in a potassium alum-diphtheria toxin precipitate which lead to an increase in the immune response to the toxin [98]. Potassium alum was later substituted for aluminium phosphate or aluminium hydroxide, and although not following the chemical nomenclature, these substances were also depicted "alum" [96]. Today most alum based adjuvants are provided in an aluminium hydroxide or aluminium phosphate gel, since they quickly adsorb antigens and are made in a standardized way [96] which makes results easier to validate.

How does it work?

An important physicochemical characteristic of aluminium adjuvants is the particle size, which is comparable to microorganisms, making phagocytosis by antigen presenting cells (APC) possible [96]. The theory of the adjuvant depot effect, in which the adjuvant slowly releases the antigen to keep the immune system alert, may be of importance for the effect of aluminium adjuvants. However, alum has potent immunostimulatory direct effects not coupled to a depot effect. Kool *et al.* showed that alum-induced uric acid release from apoptotic cells trigger dendritic cells (DCs) to initiate a Th2 associated immune response, which was abolished upon removal of DCs [99]. In contrast, macrophages and mast cells were shown not to be essential for the alum supported induction of T and B cell responses [100], indicating that alum also can initiate APC independent adaptive immune responses. Thus, alum is a multipotent stimulator of the immune system. However, since alum is associated with a Th2 immune response, introducing alum in vaccines to induce a Th1 response is counteractive, as shown by immunizing mice with a tuberculosis vaccine [101].

Health issues

Introducing aluminium containing substances in the body may raise questions about toxicity when spread into circulation and tissues. Indeed, already 1 hour after intramuscular injection of a radioactive aluminium isotope, the substance was detected in blood and excreted urine [102]. However, the body is constantly exposed to aluminium via ingestion of food and liquid. The total daily intake of aluminium was calculated to 5-10 mg in humans which would be excreted via urine in healthy subjects [103]. Together with the long use of alum in vaccines, the risk of intoxication is considered small. It might be added that mice receive a comparably

larger depot of alum upon immunization compared to humans. This approach has however been used for a long period of time without known adverse effects.

In our hands

The use of aluminium hydroxide solution in paper 1 and 2 followed the instructions from the manufacturer. Briefly, the alum solution was added drop wise with constant mixing to either PBS (paper 1) or the cBSA carrier (paper 2). This was to ensure proper mixing of the alum and cBSA to give a combined stimulation of the immune system upon injection.

Cationized bovine serum albumin (cBSA)

Bovine serum albumin (BSA) contains several immunogenic sites, which are distributed along the entire protein, although the highest antibody affinity is found in the COOH-region [104]. In a study by Dosa *et al.*, some peptides of BSA was shown to induce production of anti-BSA antibodies when injected into mice and some induced a suppressive effect [105]. However, in later studies, Muckerheide *et al.* reported that cationization of native BSA increased the immunogenic properties of BSA, due to an induced attraction of the positively charged cBSA to the negatively charged cellular surfaces on APCs [106].

We have used cBSA coupled to peptide antigens in previous studies [44] and in paper 2 we wanted to elucidate the anti-atherogenic effect of cBSA alone. Thus, two doses of cBSA were mixed with alum according to manufacturer's instructions. The lower cBSA dose was the same as used in previous studies and the higher was doubled in concentration. The rationale for using cBSA in this study was to initiate a suppressive immune response to counteract atherosclerosis development.

Immunization strategy

Injection strategy

Mice were immunized with either alum or PBS alone (paper 1) or alum mixed with cBSA in 2 different concentrations (paper 2). In both studies, the reagents were injected subcutaneously in the neck region. According to a common theory, tissue resident APCs would pick up the injected antigen and transport it to the draining lymph nodes. The head and neck tissue drains to cervical- and facial lymph nodes in the rat [107] and this is the predicted site for antigen presentation to T cells. This immunization strategy was chosen due to the practical simplicity and high success rate

and it is relatively painless [54, 108]. It is also the most commonly used injection method. The absorption rate is also slower than reported from intramuscular and intraperitoneal injections [54]. Briefly, the injection occurred as follows. After sedation to immobilize the mouse, loose skin in the neck was raised and the needle was inserted perforating the skin. A volume of 100 µl of fluid was injected to spread in the subcutaneous space, the needle was removed and the skin lowered. A few seconds later the mouse became conscious and was returned to the cage. In paper 1, mice were immunized at 6, 9 and 11 weeks of age and in paper 2 at 25, 27, 29, 31, 33, 35 and 36 weeks of age. The reason for the discrepancy between the strategies used in paper 1 and paper 2 is the different hypotheses. In paper 1 the immunization strategy would elicit an immune response before initiation of hypercholesterolemia. In paper 2 the mice were hypercholesterolemic and had lesions at the time of the first injection and the strategy was to counteract already manifested atherosclerosis. The 3-time biweekly immunization strategy in paper 1 was also used in a similar immunization study [109]. In both studies this proved to be an efficient method of inducing regulatory T cells. In a similar approach repeated daily injections of ovalbumin during a 2-week period also induced regulatory T cells [110] and we expanded this design in paper 2 to 7 biweekly immunizations with the goal to induce regulatory T cells. The increased amount of immunizations in paper 2 vs. paper 1 was due to the different age and atherosclerotic burden in these systems. We reasoned that the immune system of the mice in paper 2 was less responsive due to increased age as well as exaggerated atherosclerosis and therefore needed additional stimuli to exert a protective response. In a previous study using antibody therapy on *Ldlr*$^{-/-}$*Apobec1*$^{-/-}$ mice treatment started at 25 weeks and stopped at 29 weeks of age [111]. Since active immunization is considered to require a longer time to generate an immunological response [112] we prolonged the study period in paper 2 to 11 weeks from the first immunization.

Diet strategy

In paper 1, mice were given a chow diet from birth until death at 12 weeks of age. The group that was euthanized at 25 weeks of age received chow diet from 3-4 weeks until 10 weeks of age, when diet was shifted to a high fat diet that was maintained until death. This diet strategy was designed to induce disease after initiation of a protective immunization. In contrast, mice in paper 2 received a high fat diet from 4 weeks of age to 24 weeks of age to induce an advanced plaque phenotype. In order to mimic the clinical situation were patients receive nutritional advice and statin treatment, mice were transferred to chow diet at week 24 which was maintained until death at 36 weeks of age. Here the objective was to study an intervention in mice with manifest atherosclerosis but reduced plasma cholesterol levels.

In paper 3, mice received chow diet until 10 weeks of age, when it was shifted to a high fat diet which was kept until death at 14 or 18 weeks of age. The rationale behind this diet design was to study the initial immune response to hypercholesterolemia during the first 8 weeks of diet.

The diet design of paper 4 was based on the findings in paper 3 with some adjustments. Mice were shifted from chow to high fat diet at 6 weeks of age and continued to receive this diet until death at 14 weeks of age. This corresponded to 8 weeks of high fat diet which was chosen in order to obtain a higher, more easily assessed lesion size but still study the early immune response to hypercholesterolemia.

Surgical procedure at euthanization

Mice were anesthetized by use of Isofluran (Forene, Baxter) but since the induction period is short mice were further given an injection of a mixture of Xylazine (Rompun, Bayern Healthcare) and Ketamine (Ketalar, Pfizer), which gives a combined anaesthetic and sedative effect. The animals were immobilized within minutes and were not allowed to retain consciousness again. Using scissors and forceps the peritoneum was removed and the thoracic cavity was revealed. The tip of a needle was allowed to enter the right ventricle of the heart to remove blood while the heart was still contracting. The average amount of blood gathered was 0.5-0.8 ml depending on the size of the mouse. The remaining plasma fraction present after centrifugation was usually sufficient for the plasma lipid, cytokine and antibody measurements later assayed. Next, the spleen was removed and stored in PBS prior to single-cell preparations. The saphenous artery was cut at the lower left hind limb and the heart was perfused with PBS that emptied the cut saphenous artery and remaining arterial blood. In paper 3, mediastinal, brachial, axial, renal, iliac, sacral lymph nodes and thymus were removed and stored in PBS and in paper 4 the mediastinal lymph nodes only were isolated. An anatomical orientation to these lymph nodes is found in reference [107]. Next, in paper 1-4, the circulatory system was perfused with Histochoice (Amresco) via the heart-saphenous artery route to fixate the tissue. In paper 1-2, scissors and forceps was used to remove the tissue lining the descending aorta and the aorta was cut longitudinally along a midline and cut transversally at the aortic arch. The aorta was then mounted *en face*, lumen side up on ovalbumin (Sigma) coated glass slides and stored in Histochoice. Finally, in paper 1-4, the heart was isolated and stored in Histochoice.

Cell based procedures

Single-cell preparation

The spleen is a major immunological organ and relatively large, which makes it the ideal organ to isolate cells for various immunological tests. Cell based assays require separation of the tissue resident cells into a single cell suspension. Depending on the cell type of interest, different isolation methods may be used. In paper 1-3, the primary objective was to study T cells and since the majority of cells in spleen are T cells the spleen was meshed through a 70 µm nylon filter to obtain a single cell suspension. In paper 4 part of the objective was to obtain $CD11c^+$ cells which are present in smaller numbers in spleen. Therefore, the spleen was pre-treated with digestion enzymes to enrich the $CD11c^+$ cell fraction in the single cell suspension. We used a preformed digestion mixture from Stemcell Technologies containing Collagenase D and DNase I to pre-treat the spleen before mesh through the nylon filter. Similar digestion mixes are used in DC enrichment protocols found in the *Current protocols in Immunology* and *Dendritic cell protocols* [113, 114]. Collagenase degrades native collagen making the organ more permeable which increases the cell yield in the mesh process. DNase I degrade free DNA released from cells that may get damaged in the collagen degradation process that would otherwise form aggregation of cells "glued" together by free DNA.

In paper 1-4, meshed cells were washed in PBS or nutrient medium (RPMI-1640) and prepared for flow cytometry analysis or cell culture. If the objective is to study leukocytes, the erythrocytes have to be removed from the cell suspension. This was performed by incubating the cells in an ammonium chloride buffer which lyses the membrane of erythrocytes earlier than the leukocytes. The buffer treatment was optimized to obtain a large amounts of leukocytes compared to erythrocytes. In paper 4, cells were not treated with lysis buffer due to a possible interference with the magnetic cell isolation used in later stages of cell isolation.

Cell isolation using magnetic beads

In paper 4, the isolated splenocytes were further divided into $CD11c^+$, $CD4^+$ and $CD8^+$ cells using a magnetic cell sorting system provided by Stemcell Technologies. Briefly, cell specific antibodies were added to the cell suspension and allowed to adhere to target sites. Magnetic particles coupled to a secondary antibody directed towards the first antibody were added and a conjugate between the target cell and the antibody-magnetic particles was formed. Next, the cell suspension was put into a strong magnet which attracted the magnetically labelled cells to the sides of the suspension tube. The $CD11c^+$ and $CD4^+$ cells were positively isolated, meaning that

these cells were magnetically attached to the tube upon decantation of the cell suspension containing the unlabelled cells. In contrast, the $CD8^+$ cells were negatively isolated. Thus, the cell suspension was incubated with antibodies directed towards CD4, CD11b, CD19, CD45R/B220, CD49b and TER119, which are considered non-$CD8^+$ cell markers. Consequently, upon decantation the non-labeled cell population was removed and saved for further culturing. From each spleen, $CD11c^+$ cells were first isolated and the decanted $CD11c^-$ cells were subsequently used for $CD4^+$ cell separation. Again, the non-labelled fraction from this step was used for the negative isolation of $CD8^+$ cells. By using this method the risk of contaminating the $CD8^+$ cell fraction with $CD8^+$ DCs expressing CD11c was minimal.

Proliferation of Splenocytes

In vitro cell proliferation assays are considered to give a measurement of the priming status of cells in the *in vivo* system prior to cell isolation. Cells that initiate a higher DNA synthesis rate and subsequent proliferation upon stimulation with a mitogen *in vitro* originates from an *in vivo* environment were they have been primary activated. Thus, cells that have a higher proliferation are derived from a more activated immune system, responding to an infection or endogenous inflammatory stimuli. The extent of proliferation in T cells also regulates the cytokine repertoire and cell differentiation [115, 116]. The choice of mitogen depends firstly on the cell type to be studied and usually APCs or T cells are the target cell type. Secondly, some mitogens are chosen in order to initiate activation of a certain target cell population with documented role in disease. If the specific mitogen is unknown, broad spectrum mitogens are used which usually give a less distinct response, with proliferation of several different target cell populations. Since an effective specific T cell mitogen connected to atherosclerosis is not yet found we used Concanavalin A (con A) to induce proliferation. Con A is a lectin, originating from Jack-bean (*Canavalia ensiformis)*. It has been used in T cell proliferation assays since the 1970s and is reported to stimulate T effector cells, cytotoxic T cells and T suppressor cells [117]. In paper 1-4, we stimulated splenocytes with con A during 72 hours in 37° C and 5% CO_2, according to a widely used protocol and measured the extent of proliferation with any of the 2 techniques described below.

^{3}H-Thymidine incorporation

In this technique, radioactive thymidine is incorporated into the new strands of chromosomal DNA upon cell division. Thus, the amount of incorporated thymidine increases with the rate of proliferation. The cell cultures were stimulated with con A for 72 hours and radioactive thymidine was added to the cell cultures during the last 16-18 hours of proliferation before terminating the incorporation by freezing the cell

culture. The cells were later harvested on a membrane and incubated with scintillation fluid which absorbs the energy emitted by the radioactive isotopes and emits light that is detected by a scintillation counting machine. In paper 1, 2 and 4 we used the thymidine incorporation technique to monitor the proliferation of splenocytes (paper 1-2) and of isolated $CD11c^+$:$CD4^+$ and $CD11c^+$:$CD8^+$ cocultures (paper 4).

CFSE incorporation

Carboxyfluorescein succinimidyl ester (CFSE) is a fluorescent dye that incorporates intra-cellularly. It is useful in proliferation assays due to its progressive halving between daughter cells upon mitosis which can be monitored in a flow cytometer [118]. CFSE labeled cells can be monitored both *in vivo* and *in vitro* and in the *in vitro* proliferation assay up to 5 detectable cell divisions may be detected. An advantage with this method is the possibility to include fluorescently labeled antibodies to characterize the cell type, cytokine receptors, cytotoxic activity, activation markers and cytokine production in the CFSE labeled cells [119]. In contrast to ^{3}H-Thymidine incorporation, CFSE is added at the start of con A-induced proliferation which is allowed to continue for 4 days before staining with additional antibodies and analysis by flow cytometry. In paper 3, we costained CFSE labeled cells with the T cell markers CD3, CD4 and CD8 to detect cell type specific proliferation patterns. We were not able to detect stable peaks in CFSE fluorescence depicting each cell division but analyzed a pattern with more variable peaks, probably reflecting cell division at different times. Due to this, the cut-off for proliferation was set at "number of cells that had divided at least one time". Given this, one has to keep in mind that this reflects a basic activation in response to antigen stimulation. Analysis of several cell divisions is a more definite method with the capacity to detect activiation of specific memory cell populations. However, since we stimulated the cells with con A, the interest was in detecting general differences in proliferation based on the diet interventions.

Cytokine analysis

In order to monitor the cytokine production in cells activated by con A we used multiplex analysis kits from Meso Scale Discovery which detected 7 or 9 different cytokines in cell culture medium. The cell cultures used for cytokine analysis were stimulated for 72 hours before being terminated by freezing at -80° C. The cytokine analysis kits are constructed as 96 well plates were each well contains spots lined with capture antibodies specific for each of the cytokines included in the 7 or 9-plex assay. Plasma or cell culture medium was added and cytokines therein attached to respective capture antibody. Next, anti-cytokine antibodies labelled by an

electrochemiluminescent compound was added and covered by a buffer which will enhance chemiluminescence. The plates were loaded into a machine that applied a current onto the electrode that covers the bottom of the plate. This caused the labelled compound to emit light which was detected and amplified by the machine to exert a quantitative amount of the cytokine.

This method has an advantage compared to ordinary ELISAs in that there are no washing steps and that a multiple array of cytokines can be analyzed at once. According to the company the method is precise in that the background signal from non-specific binding is kept low. This was also the result when used in our hands.

Cytokine measurement of plasma and/or cell culture medium was used in paper 1-4.

Flow cytometry

The detection system

A large part of the data analyzed in the thesis manuscripts are derived from cells stained with fluorescently labelled antibodies and acquired by flow cytometry. The technique is based on (laser) light which upon encountering a solid matter is reflected onto detectors that reproduce the object morphology according to the reflection pattern. Cells have to be present in a single-cell form to achieve this detection of cell size and granularity. Based on these parameters, the software program in the machine plots cells on a graph which allows cells to be roughly distinguished as lymphocytes or granulocytes. In order to characterize cell populations further, cells are pre-incubated with fluorescently labelled antibodies directed towards extra- or intra cellular proteins which are considered unique to certain types of cells. The flow cytometer we use is a Cyan ADP (Beckman Coulter) which is equipped with 3 lasers that emit light of different wave lengths. Antibody labelled cells are applied onto a fluid stream that forces them through a narrow channel were they are exposed to the released laser light. The fluorescent compound is exited and emits light which is identified by ideally 1 out of 9 different detectors, which signals to the software program to plot a dot positive for that detector. In reality, the emitted light of some fluorescent compounds are detected by several detectors, which blurs the analysis. Anyway, in the same sample there is a possibility of combining up to 9 different fluorescent compounds which can be distinguished by the detectors. Since the fluorescent compounds are bound to different antibodies, one can analyze up to 9 different target molecules in the same cell. Some fluorescent compounds give rise to a strong signal through a specific detector, whereas others give rise to a weaker signal submitted equally via most commonly two different detectors. Due to the risk of contamination of emitted light onto several detectors we constructed the antibody panels so that

antibodies targeting proteins that are found in vast amounts on the target cell were equipped with weaker fluorochromes while antibodies targeting less abundant proteins were tagged with strong fluorochromes. In this way different cell populations were monitored with relatively high efficiency. However, with increasing amounts of fluorochromes there is an increased complexity in separating the detected signals and because of this the largest amounts of fluorochromes used in a single sample was 8.

The labelling system

We used monoclonal antibodies labelled with fluorochromes (Biolegend or eBioscience) which were produced in mouse, rat or hamster. Briefly, the cell suspension of 2-4 x 10^6 cells obtained in the cell isolation procedure (single cell preparation) or blood cells were incubated with antibodies targeting the Fc-receptor part of already cell bound antibodies. This reduces the unspecific binding of the antibody-fluorochrome complexes targeting extra cellular molecules that are applied in the next step. Subsequently, the cell suspension was washed with buffer to remove unbound antibodies and cells were resuspended in a salt buffer. If incubating in blood, erythrocytes were lyzed after the antibody incubation and cells were resuspended in the salt buffer. In most studies intra-cellular proteins were also targeted by continuing the extra-cellular protocol with a fixation step were cells were permeabilized and fixated in a steady state. Cells were Fc-receptor treated and subsequently incubated with antibody-fluorochrome complexes followed by washing and resuspension as for the extra-cellular staining.

The analysis system

Cells were characterized using a gating system starting with a rough isolation of the cell population of choice based on size and granularity in a dot plot graph. Lymphocytes constitute a fairly condensed cell population on such a "forward scatter – side scatter" plot whereas antigen presenting cells are more spread out. Secondly, cells were gated on major extra cellular cell markers associated with T cells or DCs. Subsequently, the gated cells were further gated based on expression of cell markers specific for the cell population to be analyzed, using dot plot graphs or histograms. In most manuscripts a negative control and a FMO (fluorescence minus one) control was used to distinguish the positive signals from non-specific signals generated by background noise. The FMO constitute all antibodies present in the antibody panel except one. Accordingly, the detector signal for the excluded antibody should be low or zero whereas signals for the other antibodies should be normal. The noise limit was put by setting a gate were less than 1% of the cells in the FMO control were positive. Thus, positive cell populations in the samples to be analyzed were plotted within the gate which should contain a minimum of false positive cells. Fixation of cells give rise

to increased background noise probably as a result of neo-epitope antibody binding which may be troublesome to distinguish from positive signals of antibodies bound to especially less abundant proteins. In most cases, the FMO control would help to gate the true positive signal but in some cases gates were put according to what seemed biologically valid for such a population based on prior knowledge on the properties of the population.

Flow cytometry analysis was performed on cells in study 1-5.

Staining of tissues

In order to monitor the atherosclerosis development, isolated aorta and hearts were stained for lesion content, neutral lipid, collagen, macrophages and T cells. Several tissue staining techniques were used as explained below.

En face quantification of aortic lesions

This technique was reported for analysis and quantification of whole aorta segments by Tangirala *et al.* [120]. Briefly, the descending aorta is opened up longitudinally, pinned up on black wax and stained with a lipid stain. This allows a gross overview of lesion severity and location in the aortic tree. Brånén *et al.* [121] modified the technique allowing the aorta to be placed on a glass slide for microscopical examination and we use this method to quantify the total lesion size in the descending aorta using a software program. This technique is particularly useful in mice with advanced plaque structure (paper 2), whereas the lesion size of mice in paper 3 and 4 were too small to be analyzed with this method.

Analysis of subvalvular lesions

This method allows for quantification of lesion size and has several other applications like quantification of collagen content within the lesions as well as quantification of intra-lesional cells. As described by Paigen *et al.* [89], lesions can be quantified by sectioning of the heart in the subvalvular area were initial growth of lesions occur. We used 10 µm thick sections that were applied on a SuperFrost Plus slides (Microm) that adheres to the section via electrostatic attraction. The hearts were stored in Histochoice (Amresco) which preserves the plaque structure that is easily visualized in a microscope after sectioning.

Staining of collagen and neutral lipids

In order to stain intra-lesional neutral lipids, sections were incubated with an Oil Red O-isopropanol solution and sections were scanned to produce a digital picture. Using a computer software program, the Oil Red O stained lesions were marked and the stained area computed, a technique originally adopted from Paigen *et al.* [89]. To stain collagen deposits in the plaque, Masson's Trichrome stain (originally developed by C.L. Pierre Masson [122]) was used. It essentially distinguishes cells from surrounding connective tissue. In atherosclerotic lesions it is used to detect collagen threads between cells. High collagen content within the lesion is associated with a stable plaque phenotype whereas lesions with low content have a higher risk to rupture. First, sections were fixated with Buin's solution followed by staining of cytoplasma, muscle fibres and keratin with Biebrich's scarlet-acid fuchsin solution. Secondly, sections were incubated with a phosphomolybdic-phophotungstic acid solution which is thought to increase binding possibilities to collagen in the tissue, either by blocking non-collagen epitopes [123] or by enhancing binding to the collagen epitopes [124]. Next, aniline blue solution was applied which stains the collagen and mucus tissue. In order to preserve the tissue, sections were incubated in increasing concentrations of ethanol followed by excessive ethanol removal by Xylene. For quantification of collagen content in the lesion we applied the same computer assisted technique as for Oil Red O staining.

Oil Red O staining of lesions was used in paper 1 and Masson's Trichrome staining was used in paper 2.

Immunohistochemistry

In order to quantify the content of specific cell types or antibodies directed towards lesion antigens we applied target specific antibodies on the tissue using immunohistochemistry. Briefly, the tissue was washed with buffered salt solution to remove tissue debris and further cleared of H_2O_2 deposits that may disturb antibody binding. The tissue was incubated with serum from the species in which the secondary antibody was produced, in order to prevent non-specific binding of the secondary antibody. The primary antibody was diluted in buffered salt solution and allowed to adhere to target molecules in the tissue, either over night at 4° C or for 1 hour at 37° C. The biotinylated secondary antibody was applied and allowed to incubate for less than 1 hour followed by washes in salt buffer to remove unbound antibody. In some assays (IgM and IgG, paper 3) the primary antibodies were biotinylated and then no secondary antibody was used. The biotin was coupled to

avidin according to a method developed by Hsu *et al.* [125] (we used the Vectastain ABC elite kit, Vector Labs) allowing complex formation. A chromogen was added, 3,3' diaminobenzidine (DAB), which reacts with the ABC complex generating a brown colour which was developed over a time period of 1-20 minutes. The development process was stopped by putting the slides in distilled water and the cell nuclei were stained with hematoxylin to aid morphometry analysis. In order to preserve the stained tissue the sections were incubated in increasing concentrations of ethanol followed by Xylene treatment to clean excessive alcohol from the tissue.

Using this technique, subvalvular sections were stained with primary antibodies targeting MOMA-2 (macrophages, paper 1-4), CD3 (T cells, paper 1-2), oxidized LDL (paper 1, 3), apolipoprotein B, IgG and IgM in paper 3. The oxLDL antibody was specific for the malondialdehyde (MDA)-modified 661 to 680 amino acid sequence of human Apo B and a gift from BioInvent International AB, Lund, Sweden.

Analysis of plasma

Cholesterol and triglycerides in plasma

For detection of triglyceride and cholesterol content in plasma we use a ready-made kit (Infinity triglycerides reagent and Infinity cholesterol reagent, Thermo Electron). The triglyceride kit is based on a method by Wako Pure Chemical Industries that was modified by McGowan *et al.* [126] and Fossati *et al.* [127]. Briefly, it is based on the finding that triglycerides are hydrolyzed by lipase to free fatty acids and glycerol. The glycerol is phosphorylated in a reaction which also produces hydrogen peroxide as by-product. The hydrogen peroxide is used as a reactant in a Trinder type colour reaction [128] which produce a red dye possible to detect in a plate reader measuring absorbance at 492 nm.

The cholesterol kit is based on the method of Allain *et al.* [129] were cholesterol esters are hydrolyzed to free cholesterol that is oxidized in a reaction were hydrogen peroxide is formed as a by-product. As in the triglyceride assay, the hydrogen peroxide acts as a reactant to generate a chromophore out of two otherwise inert compounds which can be quantitated spectrophotometrically at 492 nm.

Plasma cholesterol and triglycerides were quantified in paper 1-4.

Antibodies in plasma

Total IgA, IgM, IgG, IgG1, IgG2a, IgG2b and IgG3 in plasma was quantified with a kit from Meso Scale Discovery were 96 well plates coated with capture antibodies specific for all antibody subtypes were allowed to bind to target antibodies in the added mouse plasma. The technique was as described in the cytokine analysis section. Measurement of the total antibody concentration in plasma gives information on I) major deviations in antibody production after an intervention and II) if the IgG1/IgG2a/c balance associated with a Th1/Th2 balance is shifted.

Measurement of the total antibody levels in plasma was used in paper 4.

Mouse plasma IgM and IgG antibodies directed towards native LDL (paper 1), copper-oxidized LDL (paper 1 and 3), peptide 210 (paper 1), ApoB immune complexes (paper 2) and MDA-p210 (paper 3) were detected using enzyme-linked immunosorbent assay (ELISA). This technique resembles the technique used by Meso Scale Discovery, with the difference that each well of a microtiter plate contained capture antibodies for a single antigen. Either the antigen was seeded first and capture antibodies added in a second step (indirect ELISA) or the capture antibody was lining the bottom of the well with the antigen added in a second step (Sandwich ELISA). Further, since the risk of unspecific binding is higher than in the Meso Scale method, unlabelled material was removed by extensive washing steps with buffered salt solution. Briefly, the indirect method was used to quantify plasma antibodies against peptide 210, MDA-p210, native LDL, and copper-oxidized LDL whereas apoB immune complexes were detected by sandwich ELISA. In the indirect method the bottom of a micro titer plate was coated with the antigen and after blocking, antigen specific antibodies in the plasma were allowed to couple to the antigen. Next, a biotinylated secondary antibody was coupled to the primary antibody followed by addition of streptavidin conjugated to alkaline phospatase, which binds to biotin and forms a complex. In the next step a chromogen was added, which reacted with the alkaline phospatase to form a yellow dye that was measured at 405 nm in a spectrophotometer. In the sandwich method ApoB specific capture antibodies were coated in the bottom of a microtiter plate and the antigen containing plasma added. Since modified ApoB particles, like oxLDL are considered non-self they will be covered by antibodies that target them for degradation via phagocytosis. Such an antigen-antibody formation is called an immune complex. Consequently, an increase in modified ApoB particles will also give rise to an increased amount of ApoB immune complexes. Tertov *et al.* showed that the major part of ApoB content in the immune complexes was LDL [130]. Further, removal of IgG and IgM from the serum of coronary heart disease patients lowered the serum atherogenicity when

cultured with unaffected human aortic intima [131], indicating that ApoB immune complexes are associated with atherogenicity of the serum in humans. The next step in the ApoB sandwich ELISA was addition of biotinylated antibodies targeting mouse immunoglobulins and biotin-streptavidin-chromogen formation was as for the indirect method. In both methods, secondary antibodies targeting mouse IgM, IgG, IgG1 and IgG2c was used to I) characterize the immune response between the natural and induced humoral immune system and II) the Th1 (IgG2c) vs. Th2 (IgG1) balance in the response.

Protein analysis

Western blot

We used western blot to detect oxLDL in the gelatinous tissue formed at the injection site of mice in paper 1. Briefly, the tissue was homogenized and precipitated, loaded and run on a SDS-PAGE gel together with purified native and copper-oxidized LDL, transferred to a membrane which allowed an oxLDL specific antibody to couple to target antigens in the loaded material. The oxLDL antibody was specific for the malondialdehyde (MDA)-modified 661 to 680 amino acid sequence of human Apo B and a gift from BioInvent.

Statistical analysis

In order to validate if the quantified values in two experimental groups differed we applied unpaired Student's *t*-test in groups of similar size that followed normal distribution around the mean value. Since the number of subjects rarely exceeded 15, the D'Agostino and Pearson omnibus normal distribution test failed. Thus, the use of unpaired Student's *t*-test was based on an assumed normal distribution as seen in a scatter plot. If the distribution did not appear to follow normal distribution Mann-Whitney test was applied. We decided to apply a significance level of 0.05, i.e. we allowed 5 % of the results to occur by chance.

Kruskal-Wallis test followed by Dunn's post hoc test was used to analyze plasma cholesterol and triglycerides in paper 4. Since the values of 4 different groups were statistically validated for equality and that the values did not follow normal distribution, we decided to use this rank sum test of median distribution between the groups. A significant value in the Kruskal-Wallis test suggested rejection of the null hypothesis that medians between groups were equal. Subsequently, Dunn's post hoc test assigned group wise comparisons to determine if medians were equal or diverged.

Graph pad prism 5.01 (Graphpad software) was used to analyze the data, except for paper 5 where SPSS 17.0 (IBM) was applied.

Paper 5 – Study population

The study population in paper 5 consists of a cohort from the cardiovascular part of the Malmö Diet and Cancer (MDC) study. The whole study contains 28 098 participants, living in the Malmö region, who were examined between 1991 and 1996. It is a prospective case-control study in 45-64 year-old women and men and has an overall goal in elucidating if western diet and life style is associated with a higher risk of developing cancer and/or cardiovascular disease [132]. Thus, the participants were followed for 15 years to monitor incidence of and mortality in cancers and cardiovascular disease. Apart from initial data like food intake, life-style patterns, heredity, occupational status, socio-economic factors, previous and current diseases, symptoms and medications, a blood sample was drawn. Mononuclear leukocytes were isolated from 5752 subjects (the cardiovascular cohort) participating in the MDC baseline investigation between 1991 and 1994. Viable lymphocytes, granulocytes, erythrocytes and plasma/serum was frozen and stored at -140° C. This method was successful in 99% of the participants [132]. A detailed methodological description of blood markers was performed by Pero *et al*. [133]. The viability of the cells when thawed more than 15 years later (2009) was >90% as assessed by 7AAD viability stain.

One of the goals of this programme was to stimulate future research in new prognostic biomarkers of cancer and/or cardiovascular disease. Since white blood cell count has recently been associated to mortality in cardiovascular disease [134], we used the MDC study to elucidate the association between $CD8^+$ T cells and cardiovascular risk factors as well as acute cardiovascular events (myocardial infarction or stroke). Seven hundred subjects were randomly selected from the cardiovascular cohort of the MDC study and using the Swedish Discharge Registry, the Stroke Register of Malmö and the Cause of Death Registry of Sweden we identified 139 subjects that had suffered from an acute cardiovascular event (84 CVD and 55 ischemic stroke cases) until December 31, 2008. A CVD event was defined as a fatal or nonfatal MI (i.e. ICD-9: 410), fatal or non-fatal ischemic stroke (ICD-9: 434), or death attributable to underlying CHD (ICD-9: 412 or 414), whichever came first. Cases had higher baseline plasma levels of fasting glucose, increased waist-hip ratio, systolic blood pressure and a higher incidence of diabetes, hypertension and smoking as well as lower HDL cholesterol compared to the incidence-free control subjects. The first task was to study the relationship between $CD8^+$ T cell subsets and intima-

media thickness (IMT), which is a marker of atherosclerotic lesion size. The IMT parameters used were based on 1) the mean IMT of several measurements of the common carotid artery (CCA IMT), 2) a calculated area of the lesion using IMT mean and lumen size (CCA IM) and the area of lesions at the interna/externa CCA bifurcation (BULB IMT). The correlation was analyzed by bivariate correlation tests and multivariate linear regression. In the second task the $CD8^+$ T cell level were compared in cases and controls using Students *t* test.

Results

This section summarizes the most important findings and conclusions from each paper. The complete version of each paper is present in the last section of this thesis.

Paper 1

Biological question addressed

Does alum protect from atherosclerosis?

Most important finding(s)

Alum immunization of hypercholesterolemic *Apoe*$^{-/-}$ mice reduces atherosclerosis and induces a regulatory T cell response.

Impact in the field

Understanding the immunomodulatory effects of alum in a mouse model of atherosclerosis.

This section contains a figure in the printed version of the thesis

Paper 1. Effect of Alum on spleen T cells in 12-week-old wildtype (WT) and *Apoe*$^{-/-}$ mice. Splenocytes from Alum and PBS treated mice were analyzed with flow cytometry to determine the percentage of spleen CD4^{+} cells expressing CD25^{+}Foxp3^{+}. Representative dot plots are shown for each experiment, with cells from PBS-treated mice to the left and cells from Alum-treated mice to the right. The percentages shown are of the total CD4^{+} T-cell population.

Paper 2

Biological question addressed

Does cationized BSA (cBSA) have atheroprotective effects?

Most important finding(s)

Immunization of hypercholesterolemic ApoBec-1 / LDL receptor deficient mice with a high dose of cBSA reduces atherosclerosis. This is associated with a positive balance of regulatory T cells to effector T cells and stabilized atherosclerotic lesion.

Impact in the field

The carrier of immunomodulatory agents has anti-atherosclerotic effects in itself.

This section contains a figure in the printed version of the thesis

Paper 2. cBSA immunization reduces plaque development. Plaque areas in descending aortas of untreated (no treatment) and mice immunized with 50 (cBSA low) or 100 μg (cBSA high) cBSA. Plaque areas in descending aortas were assessed by the *en face* Oil Red O staining and the percent stained area of total aortic area was determined by computerized image analysis. $*P < 0.05$ versus no treatment and cBSA low.

Paper 3

Biological question addressed

What role does $CD8^+$ T cells have in atherogenesis?

Most important finding(s)

$CD8^+$ T cells have a pro-inflammatory profile in hypercholesterolemic *Apoe*$^{-/-}$ mice

Impact in the field

$CD8^+$ T cells may have a pro-atherogenic role in *Apoe*$^{-/-}$ mice.

This section contains a figure in the printed version of the thesis

Paper 3. Proliferation of CD4⁺ and CD8⁺ cells in spleen. Proliferation of $CD4^+$ and $CD8^+$ T cells in mice fed chow or high fat diet (HFD) at 4 weeks of diet. $***P<0.001$.

Paper 4

Biological question addressed

Does the cross presentation of antigen via MHC class I on antigen presenting cells induce pro-atherogenic $CD8^+$ T cells?

Most important finding(s)

Atherosclerosis development in hypercholesterolemic *Apoe*$^{-/-}$ mice may not be dependent on cross presentation and there is no large effect on $CD8^+$ T cell activation via this route.

Impact in the field

Cross presentation has a minor role in atherosclerosis development in animals with low infectious load.

This section contains a figure in the printed version of the thesis

Paper 4. Quantification of plaque area in the aortic root of *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Tap1*$^{-/-}$ mice fed high fat diet for 8 weeks. Each dot in the figure represents one mouse.

Paper 5

Biological question addressed

Are circulating $CD8^+$ T cells in human blood associated to intima-media thickness and incidence of cardiovascular events?

Most important finding(s)

Circulating $CD8^+CD56^-IFN\text{-}\gamma^+$ T cells were inversely associated to IMT but there were no differences in $CD8^+$ T cells in subjects that developed acute cardiovascular events compared to incidence-free subjects.

Impact in the field

A novel association between IMT and circulating $CD8^+$ T cells.

Contemplation and conclusion

Do we need more treatments of atherosclerosis?

The current treatment for people with an increased risk of myocardial infarction or stroke is statin treatment and life style guidance. Statins inhibits HMG-CoA reductase resulting in reduced concentration of plasma cholesterol by increasing expression of LDL receptors and subsequent catabolism of the LDL cholesterol [135]. Statin treatment is effective in patients with already developed cardiovascular disease (CVD) [136], whereas the efficacy in primary prevention of low- to intermediate risk subjects is debated. Statin treatment was reported to reduce risk for CVD in subjects with low LDL-cholesterol levels but high CRP levels in the JUPITER trial [137]. In contrast, a recent meta-analysis found no protective effect of statins on all-cause mortality when used as primary prevention for high-risk subjects without history of CVD [138]. Apart from a lipid lowering property, statins are also reported to exert anti-inflammatory effects, including reduction of neointimal inflammation in rabbits [139] as well as lowering of the number of inflammatory cells in mouse plaques [140]. Although this seems to be eminent properties of a perfect drug it reduces the risk of developing cardiovascular events by 30-40 % [141], leaving almost two thirds of the possible risk reduction open for other therapies.

Therefore the answer is; Yes, we need more treatments!

What would be the anti-atherogenic mechanism in such a treatment to make it an efficient supplement to statins?

The Experimental Cardiovascular Research Unit has developed two novel immune-based therapies based on atheroprotective peptides present in the ApoB100 protein [44, 47]. The first approach was conducted as an active immunization approach with the peptide coupled to a cationized BSA carrier and alum as adjuvant [44], while the second was based on a passive immunization regimen with oxLDL specific antibodies [47]. Since peptide immunized mice developed less atherosclerosis we wanted to study a possible anti-atherogenic role of the adjuvant and carrier separately. As reported in paper 1 and 2 in this thesis, both Alum and cBSA reduce atherosclerosis by promoting anti-inflammatory pathways. Both therapies are associated with a shift

in T cell response favoring regulatory T cells which is reported to be important to reduce atherosclerosis [41, 42, 142]. Interestingly, regulatory T cells are also associated with the anti-atherogenic effects seen upon immunization with alum and cBSA coupled to an ApoB100 peptide [109]. Taken together, this suggests that immunization with alum, cBSA and ApoB100 peptide, alone or as a combined treatment reduce atherosclerosis by a regulatory T cell dependent mechanism. However, regulatory T cells are not reported to populate peripheral tissues like the subcutaneous area where the injected solution first encounters immune cells. Instead, tissue resident DCs are thought to phagocytose injected antigens and migrate to a draining lymph node where antigen presentation to T cells occur [143-145]. DCs induce a tolerogenic or pro-inflammatory T cell response depending on the structure of the antigen [146] , the DC [147, 148] and stimulation from the surrounding environment [149]. In this way, antigen specific stimulation would activate regulatory T cells.

Reduction of the plasma cholesterol concentration efficiently inhibits atherosclerosis development and many reports on anti-atherogenic interventions also report this as the putative mechanism. Such a trend was seen in mice immunized with cBSA, whereas Alum immunization resulted in an increased plasma cholesterol concentration. Interestingly, immunizing mice with Alum-cBSA-ApoB100 peptide and applying the same experimental protocol as Alum immunized mice also results in an increased plasma cholesterol concentration and atheroprotective effects [109]. Thus, an atheroprotective immunization strategy is not dependent upon plasma cholesterol reduction.

Another anti-atherogenic target would be the atherosclerotic lesions. Both Alum alone and cBSA immunization leads to decrease in lesion specific $CD3^+$ T cells, indicating that T cells in lesions are generally pro-atherogenic. This is supported by immunization studies were lesional T cells were associated to atherosclerosis development [150-152]. In contrast, lesional macrophages constitute a less homogenous mechanism for athero-protection. The macrophage content in cBSA immunized mice in paper 2 decreased, whereas immunized mice with alum alone (paper 1) had increased macrophage load. Hypothetically, alum treatment may induce infiltration of anti-inflammatory macrophages. Thus, increased macrophage-mediated cholesterol efflux would decrease lesional lipids while increasing plasma cholesterol through cholesterol transport. Although lesional lipid levels in alum-treated mice were similar to controls, the increase in plasma cholesterol supports such a mechanism.

Taken together, Alum and cBSA immunizations exert their athero-protective function by favoring regulatory T cell responses and decreases T cell content in lesions. However, while alum treatment increases macrophage lesional infiltration alum together with cBSA decreases the macrophage content. Although these conclusions are based on different experimental conditions, the T cell effect is evident in both reports. Considering that statin treatment has similar effects [140, 153] the challenge in additional therapies is if they provide add-on effects due to different mechanisms or if the similar protective effects neutralize each other.

$CD8^+$ T cells: Important or not?

In paper 3 we reported a profound $CD8^+$ T cell activation associated with hypercholesterolemia whereas no such effect was found in a similar approach in paper 4.

What could be the reason for this discrepancy?

Although hypothetically similar the experiment design of paper 3 differed technically from paper 4, which may have affected the outcome. The splenocytes in paper 4 were isolated using magnetic cell sorting and thereafter stimulated with conA whereas con A stimulation of non-isolated splenocytes was performed in paper 3. Although the vast majority of splenocytes consists of T cells the T cell response could be influenced by DCs, monocytes, macrophages, B cells or NK cells. Consequently, influences from the non T cell-, non-DC population may have provoked the increased $CD8^+$ culture proliferation seen in paper 3.

Supposing that the design of paper 3 is a better predictor of the actual role of $CD8^+$ T cells in atherosclerosis;

What is the evolutionary and immunological basis for development of atherogenic $CD8^+$ T cells?

$CD8^+$ T cells probably evolved to inhibit attacks by pathogens and since chronic infections are less usual in developed countries [154] a potential underuse of these cell populations could be postulated, leading to attack of self tissues. According to this hypothesis, the $CD8^+$ T cell population would be depressed during a chronic infection and thus less prone to attack self tissues. Indeed, if an immune system is

stimulated by acute virus infections that are rapidly cleared, the development of memory T cells is increased compared to a chronically infected system [155]. Although virus peptide-specific division of $CD8^+$ T cells persist during chronic infection [156], the response is gradually diminished due to decreased IL-2 production in both $CD8^+$- and $CD4^+$ T cells [157] and loss of CD127 expression on $CD8^+$ T cells [158].

Hypothetically, the viral target molecule of one of these memory T cell populations may be structurally similar to modified self molecules and may lead to auto-immune reactions. Such a molecular mimicry occurs in the $CD8^+$ T cell mediated destruction of pancreatic β-cells leading to development of type 1 diabetes mellitus (T1DM) [159]. Here, β-cell destruction was showed to occur via perforin [160], Fas ligand [161-163] and cytokine dependent pathways [164] and as commented in several reviews, the combined effect of these is crucial to initiate disease. Interestingly, the rat-insulin promoter-lymphocytic choriomeningitis virus (LCMV) model is an established model system in T1DM, in which a virus infection is used to initiate CTL driven β-cell destruction [165]. A similar system has been developed in NOD mice [166], but whether the connection between virus infection and mimicry responses in humans are valid [167] or a result of bystander T cell activation [168] is still debated. Multiple sclerosis (MS) is another autoimmune disease where a virus infection can lead to initiation of disease and a dysregulated CTL response. Most commonly, Epstein-Barr virus infection is correlated with activation of $CD8^+$ T cells [169], but the exact mechanism is unknown as is why destruction of the brain white matter occurs. Potentially similar mechanisms as the CTL activation in T1DM may take place, where mimicry or bystander effects may augment disease. Several pathogens, including Cytomegalovirus, Herpes simplex virus, Chlamydia pneumoniae and Helicobacter pylori have been associated to induction of atherosclerosis [170]. Due to the heterogeneity in pathogen properties, multiple associations to atherosclerosis have been suggested. These include stimulation of smooth muscle cell (SMC) proliferation and migration to the neointima, inhibition of endothelial cell (EC) apoptosis, lipid accumulation and promoting endothelial dysfunction [170]. Importantly, the pathogen that initiated the immune response resulting in tissue injury does not necessarily have to be present in the target tissue. Such mimicry may be dependent on heat shock protein (HSP) release which has been associated to atherosclerosis development [167]. The eukaryotic and prokaryotic HSP structure is conserved and share similarities [171], which may lead to a self-targeting immune response that was initiated by an infection. Such a mechanism has been reported both in mice [172] and humans [173] were the infection augmented atherosclerosis. Interestingly, oral immunization of mice with recombinant Mycobacterial HSP-65, made the mice immune to atherosclerosis induction initiated by a subsequent injection of heat killed *M. tuberculosis* or high fat diet [174]. This was however not attributable to decreased

lesional T cell infiltration, which was similar to control treated mice. On the other hand, subcutaneous immunization of hypercholesterolemic C57BL/6 and *Ldlr*$^{-/-}$ mice with mycobacterial HSP-65 increased lesion development [150, 175]. Further, the amount of lesional CD8^{+} T cells in immunized C57BL/6 mice were low compared to CD4^{+} T cells, which may depict a less prominent role in the disease development. This is in contrast to T1DM and MS, where CD8^{+} T cells are the dominating T cell subset around human pancreatic islets [176] and MS plaques [169, 177]. On the other hand, CD8^{+} T cells have been reported to be abundant in human atherosclerotic plaques [178], highlighting a possible difference in mouse and human pathology.

There is an interesting association between CD8^{+} CTLs and antibodies forming immune complexes that target the same antigen. Kita *et al.* stimulated DCs with a major auto antigen of Primary Biliary Cirrhosis (PBC) which elicited a CTL response in PBMCs from PBC patients but not controls. Further, pulsing DCs with the antigen coupled to specific antibodies augmented the response [179], showing that CTLs and autoantibodies recognize the same peptide sequence and that the response magnitude is dependent on both systems. Thus, if such a response would be directed towards modified LDL molecules or other modified self molecules in the vascular wall due to for instance inflammation or necrotic cell release associated with infection, it could aggravate atherosclerosis development. Subsequently, atherosclerosis interventions with augmented antibody responses may also activate CD8^{+} T cells. Atheroprotective immunization of *Ldlr*$^{-/-}$ mice with Streptococcus pneumoniae induced a molecular mimicry response with IgM secretion from B cells directed towards phosphorylcholine (PC) epitopes of oxLDL and *S. pneumoniae* [180]. Additionally, immunization of *Apoe*$^{-/-}$ mice with PC generated similar results with increased levels of IgM and IgG antibodies directed towards PC and oxLDL [45]. Since antibody production against pneumococcal polysaccharides requires CD8^{+} T cells they may have an impact on the lesion development in these systems as well [181].

This hypothesis is of course of less importance in an animal model with minimal exposure to pathogens. The animal house were the mice in paper 3 and 4 were housed provides such an environment, with relatively rigorous hygiene standards. Consequently, the mice have not been exposed to many of the viruses they would have faced in wild life and even in other animal houses. Given that the above hypothesis is true, the CD8^{+} T cell response to hypercholesterolemia may differ between animal houses. It does not however explain the different results reported in paper 3 compared to paper 4. However, it would be of sincere interest to repeat study 4 in a less hygienic animal house to test the virus hypothesis.

In conclusion, development of self-targeting $CD8^+$ T cell populations may not be evolutionary or immunologically favoured, but rather occur in an immune system with decreased infectious burden were auto-reactive T cell clones may have an increased impact in the total T cell pool.

The jump from mouse to man

There are considerate differences between mice and humans, most evidently in size but also genetically. On the immunological level, a number of differences can be mentioned, like the balance between lymphocytes and neutrophils. Whereas mouse blood has a shift towards lymphocytes, human blood is comparably neutrophil rich [60, 61]. Thus, a possible pitfall is to extrapolate the impact of lymphocyte pathological processes in mice into the human system. However, there are also considerate similarities between the systems which are of importance when translating data from mice to humans.

Species dependent differences in immune system architecture are probably correlated to evolutionary discrepancies in diet, habitat and life expectancy. Considering that humans have a longer life expectancy than mice, development of a long lasting cell memory should be of evolutionary advantage in humans compared to mice. However, several reports show similarities in the memory T cell pool. In order to maintain such memory cell pools, it is of course important that they are formed in the first place. Moreover, they have to be kept alive by "live" signals and protected from "death" signals but still have the ability to respond to cognate antigen stimulation. Although the field of memory T cells is large and complex a number of issues may be discussed. The memory T cell pool in humans gradually increase with age, possibly through interaction with different pathogens with time but seem to decrease at very old age [182]. Similarly, the mouse memory T cell pool increase with age [183], and may share similar mechanisms behind the longevity as seen in humans. Both mice and humans have a decreased response towards new antigens with age [184, 185]. In contrast, existing memory $CD8^+$ T cell clones were favored with age, constituting as much as 80% of the peripheral $CD8^+$ T cell repertoire in aged mice and 50% of the repertoire in aged humans [186-189]. Given that $CD8^+$ and $CD4^+$ T cell clones directed towards self antigens may have an impact on atherosclerosis development one might consider two scenarios. (1) The activation of autoimmune T cell clones directed towards modified self proteins (HSPs, LDL etc.) was initiated at early age and therefore the autoimmune clones are expanded with age compared to other clones. (2) In contrast, if such autoimmune clones are not favored during the process of clonal expansion compared to other antigens, the impact of the autoimmune

clones would decline with age. I would like to give an example on how to implement these hypotheses on T cell association to atherosclerosis in humans, which may be of importance when interpreting the results in paper 5.

In favor of the first hypothesis are findings in carotid artery of old patients (mean age 61 years of age) showing an increase in intra-lesional T cell reactivity towards human HSP60. Interestingly, there was a positive $CD8^+/CD4^+$ T cell ratio and the cells were strictly oligoclonal, in contrast to the polyclonal appearance of PBMCs [37]. Similarly, lesional T cells (although predominantly $CD4^+$ T cells) in aged subjects (mean age 67 years of age) were shown to be HSP60 specific [38] or have a heterogeneous response including both *C. pneumoniae* and human HSP60 specific clones [190]. Thus, with time there exists a clonal selection favoring T cell targets associated with atherosclerosis. In favor of the second hypothesis is the finding of T cell specificity in the ARMY study of young men compared to old men in the Bruneck study. T cells isolated from peripheral blood of cases and controls (based on intima media thickness measurement of common carotid arteries) were stimulated with human HSP60 to induce proliferation. Interestingly, there was an increase in HSP60 specific T cells in the cases of the ARMY study but not in the Bruneck study compared to respective control [191]. Thus, clonal selection may be negatively correlated to atherosclerosis with time. However, given that the HSP specific clones are mostly present in the atherosclerotic lesions [37], the correlation to disease may be missed by looking on T cell populations in blood. Moreover, the antibody titer towards HSP65 was previously reported to be positively associated to lesion size at baseline in the Bruneck study [192], to be sustained over time [167] as well as correlated to progression of atherosclerotic lesions and mortality due to cardiovascular diseases [193]. Further, there is a cross reactivity in B cell epitopes between microbial and human HSP60/65 which may brake self tolerance and promote atherosclerosis [194]. Considering that $CD8^+$ T cells and antibodies forming immune complexes may have the same target [179] this favors the hypothesis that cell clones specific for modified self-molecules are maintained with increasing age.

The finding in paper 5 that circulating $CD8^+$ T cells are not associated to cardiovascular events may reflect a less prominent role for $CD8^+$ T cells in atherosclerosis. However, the total $CD8^+$ T cell population may consist of cell clones directly or indirectly associated to atherosclerosis. Thus, an enlarged impact of clones affecting atherosclerosis may not be detected if solely monitoring the total $CD8^+$ T cell population. An interesting approach would then be to test whether T cell clones specific for common modified self proteins associated with atherosclerosis are enriched in subjects with cardiovascular disease and if such a clone was apparent prospectively. Considering the difficulties in analyzing lesional T cells at baseline, the

blood based approach is the most valid despite a possibly blurred resolution. This strategy would give information if atherosclerosis associated T cell clones are accumulated with time and what impact they have on disease development. Further, by immunizing with peptide targets of the enriched clones, atheroprotective responses could be initiated, similar to the results in the mouse-based approach reported by Hermansson *et al.* [195].

Another interesting aspect of the antigen response in the aging immune system is the challenge of implementing a vaccine-based approach of treating patients with advanced atherosclerosis. Since the current knowledge on such regimens is limited I would like to give an example of a thoroughly tested vaccine-based intervention in elderly that may be applicable in atherosclerosis vaccines. The antibody response to influenza vaccination declines with age [196] which render old people more susceptible to infection. Interestingly, compared to young mice of 3 different strains, old mice showed reduced response to influenza vaccination [197] with a similar mechanism as in young versus old humans [198]. Further, experiences from influenza vaccination with the MF59 adjuvant tell us that it enhances the immune responses irrespective of age in both mice and humans [199, 200]. The immune responses in old mice given vaccine and adjuvant increased to the same level as young mice given vaccine alone, showing the impact of the adjuvant in this process [199]. If we again focus on a putative atherosclerosis vaccine, the adjuvant effect may boost the immune response in the elderly. Although MF59 has a documented adjuvant effect in vaccination against viruses [201], we (paper 1) and others [202] have shown that alum induce Th2 and regulatory T cell driven responses, which is of importance in counteracting Th1 signals that drive atherosclerosis. Indeed, since immunization of mice with both alum and cationized BSA counteract atherosclerosis progression via similar mechanisms, it is conceivable that they may support the atheroprotective effect of a vaccine candidate. Further, the results in paper 2 show that 36-weeks old mice with advanced plaques can mount a protective response with a cBSA immunization procedure to stabilize the plaques and inhibit plaque progression. This adjuvant dependent effect can be compared to the antigen specific effect of an anti-oxLDL antibody based approach in mice of similar age (29 weeks) reporting a decrease in atherosclerosis [111]. Taken together, the anti-atherogenic effects of adjuvant and an antigen specific treatment may elicit a stronger effect than separately that is applicable also in old humans.

How valid are the results presented in this thesis?

According to a previous report by Ioannidis, most published research findings are false [50]. In order to elucidate putative errors in the reports included in this thesis I would like to discuss possible pitfalls concerning study design, analysis and herd behavior.

Study design

The idea to perform a study usually has a connection to previous reports. Whether results and conclusions of these are based on "true" findings or based on a type 1 error (claiming a relationship when none exists) will affect the new study from the start. The study design of paper 1 and 2 are for instance based on previous studies by our group and others claiming a relationship between the intervention and a given effect, while paper 3 is discovery based, and paper 4 and 5 based on the findings in paper 3. Concerning paper 1 and 2, the precise timing of an intervention or a diet regimen on a given outcome is less characterized. For example, two separate experiments with identical amount of immunized substance may yield different results, depending on the time and frequency of immunization. Similarly, the scheduled duration of a certain diet and/or dietary changes may affect the outcome. Taken together, assumptions on substance efficacy may be based on study regimens that hit or missed a "therapeutic window", which may affect the design of further studies.

The majority of research groups in the atherosclerosis field use similar animal models (largely *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice) which constitute a genetically homogenous study population. There are experimental advantages with this approach, but considerate hazards if transferring the results to a heterogeneous human population. In this aspect the papers 3 and 4 are interesting, since study design seem to affect CD8^{+} T cell activation. However, there is a concordance in both paper 4 and 5 in the finding that CD8^{+} T cells have a minor impact on atherosclerotic lesion development and clinical cardiovascular events, respectively. Thus, as observations from a homogenous mouse model and a heterogeneous human model coincide, the role of CD8^{+} T cells in disease development may be similar.

A large bias in mouse studies is the testing of a small population. The subjects in each treatment group are seldom more than 20 and more usually around 10 or below. According to Altman [203] a valid model can be *statistically validated* (were the population size affect the goodness-of-fit) or *clinically validated* (with strong prognostic information supporting the hypothesis). Ideally, a study is dependent on both models to increase validity. Given this, a study with a few subjects has to have

stronger prognostic values than a study with a larger population in order to minimize the risk for type 1 errors. This is scarcely performed in mouse studies which often have a less validated discovery-oriented design.

Analysis

The development of new advanced technologies has both positive and negative implications on the study errors. First of all, computerized calculations of data quantification and statistical relationships have decreased the user assisted errors in manual calculation. However, the increasing amount of study data has also implications on error rate upon interpretation of data. Analysis of flow cytometry data is in theory clear-cut, but in many cases antibody signals interfere and negative populations is modified so that the analysis of especially weak markers is less evident. Given the complex characterization of cell populations in the current practice and the small populations, there is a high risk of reporting false relationships [50]. Papers 1-5 report associations primarily between cell phenotypes and disease, whereas the cell function is less elucidated. This largely depends on earlier characterization of cell function of respective phenotype. However, it does not take into account the plasticity of cells, were the same phenotype not necessarily has to be accompanied by the same function in different environments. An interesting example of possible misinterpretation is the $Gata3^{+}$ $Tbet^{+}$ T cell [204] with combined Th1/Th2 function. Consequently, it may easily be characterized as either Th1 or Th2 by using the most reported analysis strategies.

Statistical testing may be biased by the researcher that could favor reporting of certain relationships and disregard others. An interesting variant of this is the possibility that data giving negative relationships are more thoroughly re-analyzed in order to elucidate the reason for the result, whereas a positive result is more likely accepted. Likewise, reports on negative or contradictory results may contain fewer errors due to decreased bias.

Things to keep in mind if you are looking for the Holy Grail

Last but not least I would like to stress two subjects that are often taken for granted, due to the numerous studies that support the thesis, namely the impact of regulatory T cells and cholesterol in atherosclerosis. However, a finding is not necessarily true just because a majority of reports support it and alternative hypotheses may be more valid although with less power.

Are regulatory T cells the savior or simply shedding another anti-atherogenic effect?

Although there are confident evidence that regulatory T cells has a prominent role in reduction of atherosclerosis one has to keep in mind that scientists are prone to think surprisingly one tracked in such a "hot field". Consequently, the focus on this "cell type" constructed by a man-made nomenclature may be too rigid to give a valid reflection of nature.

This type of "suppressor T cells" were discussed already 30 years ago but were disregarded due to difficulties in finding characterizing cell markers [205]. Today, cells that express markers typical for regulatory T cells have been reported to have anti-inflammatory functions with implications in several diseases, like T1DM, MS, systemic lupus erythematosus, rheumatoid arthritis, inflammatory bowel disease, psoriasis, atherosclerosis [206, 207] and cancer [208]. Instead, the challenge now is the large plasticity that encompasses this cell type, with additional cell markers found every year. The obvious question is if such a defined cell type exists in humans, or if certain properties of the surrounding tissue and regulatory T cell signaling induce a balanced immune response. Indeed, cells with regulatory T cell characteristics can shift towards naïve/effector cell characteristics [209, 210] and vice versa [210-212] or have both [213]. This is of course a problem for the current nomenclature built on cell type characterization based on stable cell markers and cell function. Present and previous findings instead point towards a situation were plasticity is the major cell characteristics. Consequently, an intervention that induces an immune cell response with certain characteristics in one environment does not necessarily have to induce the same immune response in another environment, even though the outcome is similar. For instance, regulatory T cells in mice frequently immunized with an antigen were reported to have a minor role in $CD4^+$ T cell anergy towards the antigen, which may have been regulated by competition from other cell types [214]. Similarly, reports on several non-regulatory T cell populations with anti-inflammatory properties have been published during the years, covering B-cells, DCs [215], monocytes [216], macrophages [217, 218] and NKT cells [219]. Most studies were performed in a hypercholesterolemic environment, indicating that several cell types may exert similar balancing effects as a consequence of the imbalance caused by this environment. Thus, the hazard in focusing too much on one of these cell types is that the effect of the others is less monitored. No research lab in the world has the capacity to focus on the effect of all known cell types to a certain intervention in a single experiment. An alternate way is to share a common task between various research teams, but looking at different aspects of the intervention. Although such cooperation's are apparent I would like to see a situation were scientists following the main stream idea in a "hot field" are continuously stimulated to also focus an alternate explanations. One way would be to encourage publication of negative

results, which would probably help solving some intriguing scientific questions and possibly promote alternative theories.

"Bad" cholesterol or a powerful repair system?

In this section I will discuss the impact of cholesterol in the development of cardiovascular disease and evolutionary aspects of the lipoprotein system.

The theories on what kind of food that is unhealthy versus healthy has changed during the years, and this is primarily true for fat. In 1953, Keys [3] reported an association between cholesterol levels and mortality in cardiovascular diseases in different countries which initiated the hypothesis that cholesterol and fat intake is associated to pro-atherogenic effects. There are however a number of contradictions to this hypothesis. For instance, studies in African rural tribes with predominance in food intake of meat and fatty milk show a lower plasma cholesterol level compared to people in developed countries [220, 221]. To better predict risk of developing CVD the ratio Total cholesterol:HDL is commonly used. Interestingly, a study comparing non-westernized individuals from New Guinea and a Swedish control cohort showed similar ratio, although the New Guinea cohort had no reports of CVD [222]. Although a seemingly lower ratio was found in Brazilian Indian tribe [223], this together suggests that non-cholesterol risk factors have a larger impact on disease. The large difference in diet preferences between the isolated cohorts and western societies is not necessarily coupled to fat but rather processed food and sugar. Indeed, lowering the intake of fat does not generate lipoprotein profiles with negative association to CVD in a large part of a western study population [18, 224, 225]. None of these studies have the power of large epidemiological studies but they emphasize that CVD related risks and high plasma cholesterol contain disagreements.

Although saturated fats have been associated to CVD in some studies [24, 226], a recent meta analysis of 300 000 subjects show that there is no significant evidence for such a connection [227]. However, a meta-analysis of similar size reported that whereas HDL interventions did not reduce the risk of CVD, a reduction in LDL plasma was equivalent to a decrease in coronary heart disease events [228]. In order to elucidate the connection between plasma concentration of LDL-Cholesterol and cardiovascular disease one has to acknowledge the stimuli that formed the current apolipoprotein system. The LDL-Cholesterol pathway is more efficient than the corresponding HDL-cholesterol pathway, probably for a reason. From an evolutionary standpoint, the ability to efficiently deliver cholesterol to sites of injury ought to be of advantage for our ancestors while the last 200 years of life style changes

has created a situation of potential disadvantage for this system. Thus, an advantageous lipoprotein profile has suddenly been a disadvantage, affecting the formerly best fitted genotypes most detrimentally. Thus, the LDL-cholesterol has moved from a long-time "hero" to a "bad guy" during the last few moments of human history. From a philosophical point of view, one could speculate whether it is the "bad guy" that shape the environment or vice versa. Undoublty, the present situation would, if beneficial for reproduction, generate a shift in the lipoprotein metabolism. However, given the large possibilities to intervene this state through drug treatment, it is of course interesting to speculate whether there will be any selective pressure to develop the system. Anyhow, it is rather satisfying to note that the daily promenade decrease the risk of developing CVD in both men and women [229].

An interesting indirect critique to the dietary approach in papers 1-4 comes from Foo *et al.* [230] who treated *Apoe*$^{-/-}$ mice for 6 or 12 weeks with a low carbohydrate-high protein (LCHP) or high fat "Western" diet. Surprisingly the LCHP fed mice developed larger atherosclerotic lesions than mice on high fat diet, but no increase in infiltration of lesional T cells, macrophages or circulating oxLDL was detected. This shows that diet induced atherogenesis can be modulated by other factors than fat and cholesterol. Considering the interest in such diets for human use, it would be interesting to modify the study designs in paper 1-4 to include such a diet.

Conclusion

To conclude this section, as noted by Djulbegovic and Hozo [231], the increasing amount of results from the medical research is undoublty a mixture of false and "true" findings. Given that absolute truth is impossible to obtain, we have to continuously decide what amount of potential false findings to accept in comparison to the possible benefit of "true" findings. In this context I would like to request the reader to carefully consider seemingly convincing data, for instance by thoughtfully reflect on what you just read.

Don't trust it just because it's on paper.

Populärvetenskaplig sammanfattning

Drygt en tiondel av Sveriges befolkning lider av hjärt-kärlsjukdom som också är den vanligaste dödsorsaken. Många av de akuta sjukdomstillstånden orsakas av att en bit inflammerad blodkärlsvävnad bryts loss från sitt fäste i något av de stora blodkärlen, färdas med blodet och täpper till blodflödet i mindre blodkärl. Detta leder till syrebrist och celldöd nedströms om det blockerade kärlet, vilket kan påverka både hjärt- och hjärnfunktion negativt. Den inflammerade blodkärlsvävnaden kallas ett *aterosklerotiskt plack* och består till stor del av immunförsvarsceller, döda celler, kolesterolpartiklar och celler som reparerar den skadade vävnaden. I normala fall är placket fast förankrat i blodkärlsväggen och är förhållandevis ofarligt så länge det inte bryts loss. Eftersom plackvävnaden ofta är gulaktig kan man beskriva den som en gul strumpa som skyddar resten av kroppen mot skadliga substanser som kan läcka ut. De flesta av oss har sådana här ”kärlstrumpor” och eftersom det är svårt att förminska dem är det troligen bättre att se till att de håller längre. Precis som för vanliga strumpor gäller att kvalitet och tjocklek påverkar livslängden och eftersom kärlstrumporna ständigt slits kan man dels förbättra reparationen av dem och dels minska slitaget. Kärlinflammationen kan bero på en skada som inte läkt ordentligt och därför hela tiden stressar kroppen genom att signalera att något är fel. Detta medför att kroppen både försöker reparera vävnaden och låter immunförsvarsceller leta efter potentiella faror runt placket. I denna stressade miljö verkar immunförsvaret ta fel på vän och fiende och försöker omintetgöra kroppsegna partiklar som förändrats som en bieffekt av inflammationsprocessen. Kolesterol, som i vanliga fall är viktig för uppbyggnaden av kärlväggen, ansamlas på ett onormalt sätt i dessa områden, varvid strukturen på en av dess bärarmolekyler, *Low density lipoprotein (LDL)* lätt förändras. Detta gör att de angrips av immunförsvarsceller vilket förvärrar kärlinflammationen och ökar risken för att ”kärlstrumpan” brister. Det finns dock immunförsvarsceller som motverkar detta förlopp och sådana *regulatoriska* celler kan stimuleras att bli ännu effektivare med olika metoder.

I den här avhandlingen beskriver jag två metoder att göra detta genom att vaccinera möss på ett sätt som leder till stabilare aterosklerotiska plack till följd av aktivering av regulatoriska celler. Jag tittar även närmare på en viss typ av immunförsvarscell,

nämligen T lymfocyter som uppvisar markören CD8, och dess inblandning i inflammationsprocessen. Låt oss börja med vaccinationsförsöken.

Vid vaccination framkallar man ett skydd mot t.ex. ett virus, men det har också visat sig att vaccination av möss innehållande delar av LDL ger upphov till stabilare plack genom stimulering av regulatoriska celler. Detta beror troligen på en ökad tolerans för förändrade LDL-kolesterolpartiklar i placket vilket mildrar inflammationen och gör att vävnaden kan repareras. Vid vaccinering används ofta också bärarpartiklar och ett adjuvans. Dessa gör att presentationen av *antigenet* (t.ex. delar av LDL-partikeln) för immunförsvarsceller blir mer effektiv. I arbete 1 och 2 visar vi att vaccination genom injektion av enbart adjuvanset *Alum* eller tillsammans med en hög dos av bärarpartikeln *katjoniserat bovint serum albumin (cBSA)* stabiliserar aterosklerotiska plack eller hindrar placktillväxt i möss. Detta kan bero på att Alum tar upp förändrade LDL-partiklar vid injektionsstället och ensamt, eller tillsammans med cBSA visar upp detta för immunförsvarsceller på ett sätt som aktiverar regulatoriska celler. Dessa kan producera antikroppar som motverkar inflammationen eller hindra inflammatoriska celler genom direkt kontakt mellan cellerna. Detta medför att placket kan repareras mer effektivt och i slutändan att ”kärlstrumpan” blir mer hållbar.

T lymfocyter som uppvisar markören CD8 ($CD8^+$ T-celler) är specialiserade på att döda tumörceller eller celler som är infekterade av t.ex. virus. Det finns även rapporter som visar att $CD8^+$ T-celler av misstag kan angripa kroppsegna celler i blodkärl och bidra till uppbyggnaden av aterosklerotiska plack. I arbete 3 testade vi detta i möss som fick fet kost vilket förvärrar inflammationsprocessen. $CD8^+$ T celler i möss som fått fet kost visade sig vara mer benägna att medverka till inflammation än $CD8^+$ T celler i möss som fått en mindre fet kost. *Antigenpresenterande celler (APC)* kommunicerar med $CD8^+$ T-celler och hjälper dem att reagera mot potentiellt farliga celler eller minskar deras reaktion mot ofarliga celler. I arbete 4 testade vi den feta dieten på möss som har defekt kommunikation mellan APC och $CD8^+$ T celler, vilket innebär att mössen har färre $CD8^+$ T celler än normalt. Inflammation och plackstorlek skiljde sig inte mellan dessa möss och motsvarande möss med fullgod kommunikation, vilket visar att $CD8^+$ T celler i möss inte har så stor roll i hjärt-kärlsjukdom. Vi testade även detta i människor (arbete 5) där vi kunde visa att andelen inflammatoriska $CD8^+$ T celler i blodet hos äldre människor var sammankopplat med en markör för plackstorlek i halspulsådern. Intressant nog fanns det en större andel inflammatoriska $CD8^+$ T celler i blodet hos personer med mindre plack och vice versa. Trots detta fanns det inget samband mellan $CD8^+$ T celler och insjuknande i hjärtinfarkt eller stroke, vilket gör det mindre troligt att $CD8^+$ T celler kan användas som en indikator för hjärt-kärlsjukdom.

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