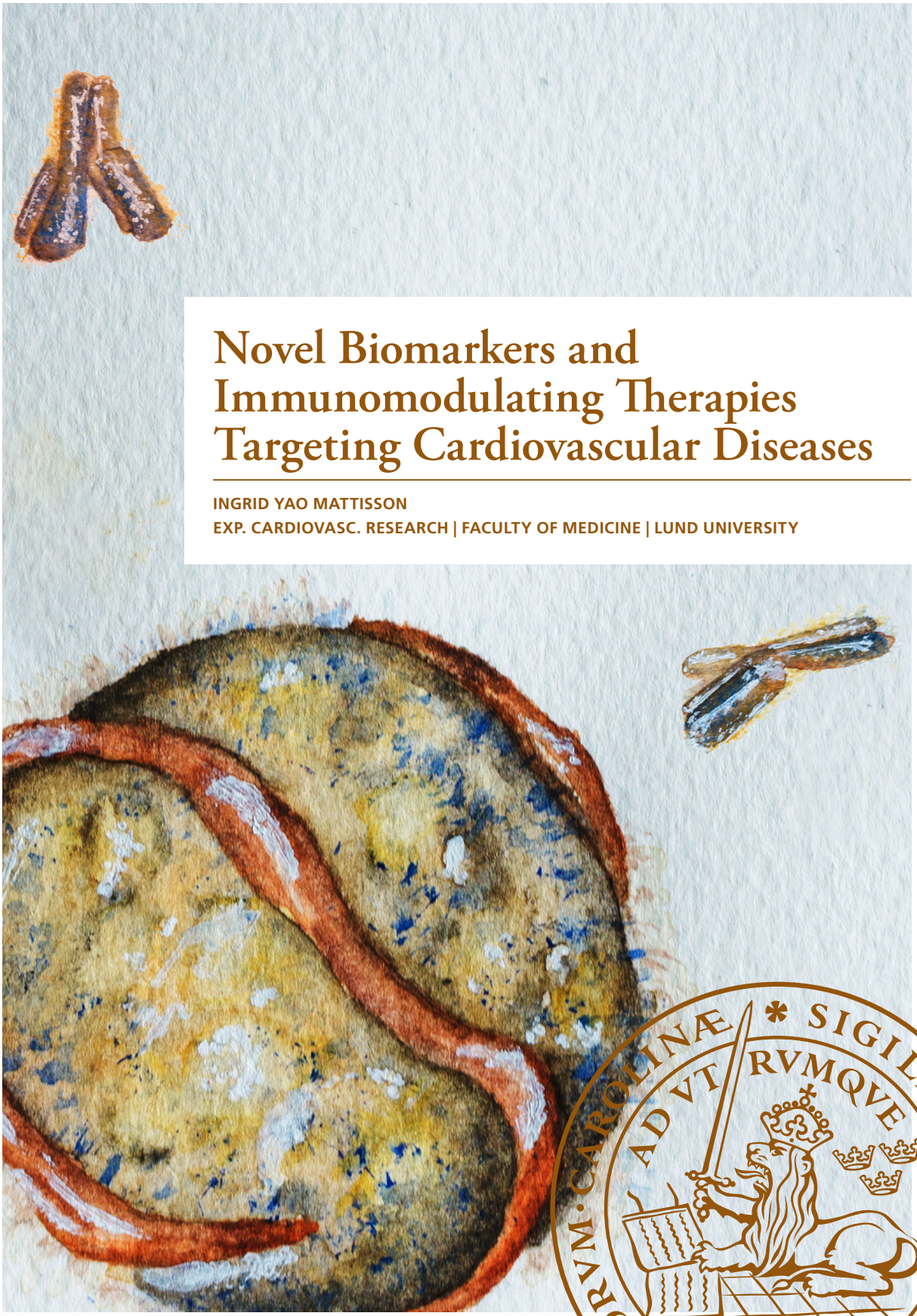
Novel Biomarkers and Immunomodulating Therapies Targeting Cardiovascular Diseases

Yao Mattisson, Ingrid

2019



Novel Biomarkers and Immunomodulating Therapies Targeting Cardiovascular Diseases

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EXP. CARDIOVASC. RESEARCH | FACULTY OF MEDICINE | LUND UNIVERSITY

INGRID YAO MATTISSON was born in Uppsala and moved shortly thereafter to Skåne. She was introduced early into the field of medical research and even before starting her own academic studies she was experienced in taking care of laboratory animals. Ingrid studied molecular biology at Lund University and did several projects after that within the field of Vascular Physiology before she started her PhD studies at the Cardiovascular Research Unit in 2015.

Ingrid successfully continued to work on newly established projects within the field of cardiovascular disease and systemic lupus erythematosus (SLE). It is always with great enthusiasm, ambition and dedication to her work that she undertakes different assignments.

In parallel to research Ingrid has a passion for her Icelandic horses, a valuable contrast to "research life". In this thesis, Ingrid describes her research findings in a clear and personal way, showing the great amount of knowledge she has gained over the last years.

-Maria Wigren, co-supervisor

Printed by Media-Tryck, Lund 2019 NORDIC SWAN ECOLABEL 3041 0903

LUND UNIVERSITY

FACULTY OF MEDICINE

Department of Clinicla Sciences, Malmö
Experimental Cardiovascular Research

Lund University, Faculty of Medicine
Doctoral Dissertation Series 2019:39
ISBN 978-91-7619-768-4
ISSN 1652-8220

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LUND
UNIVERSITY

DOCTORAL DISSERTATION

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To be defended at Clinical Research Centre room 93-09-002, Jan Waldenströms gata 5, Malmö. Friday May 3rd, 2019 at 13.00

Faculty opponent
Professor Johan Kuiper

Leiden University, Netherlands

Organization LUND UNIVERSITY	Document name **DOCTORAL DISSERTATION**
	2019-05-03
Ingrid Yao Mattisson	Sponsoring organization

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Abstract

Atherosclerosis is the most common underlying cause of myocardial infarction and stroke, responsible for 80% of all CVD-related events. It is a chronic inflammatory disease characterized by an accumulation of lipids, apoptosis and fibrotic scar tissue in the vessel wall. The immune system plays a central role in the development of atherosclerosis. Initially, the immune system protects against atherosclerosis progression by clearance of oxidized and modified lipoproteins and apoptotic cells. If the clearance of these materials fails or is not enough to resolve the inflammation, it can result in chronic inflammation and autoimmune reactions. To understand how immune responses are involved in the atherosclerosis development, it is essential to study the autoimmune mechanisms. This thesis presents studies covering immunological responses against atherosclerosis related antigens in human and murine models.

The first part addresses novel biomarkers and their association to certain CVD events. We identify circulating death receptors in the plasma and and demonstrate that these reflect the level of ongoing cell death by apoptosis. We also identify that subjects with metabolic cardiovascular risk factors have elevated circulating levels of death receptors suggesting that these risk factors cause tissue injury. Subjects with signs of increased receptor-activated apoptosis are at increased risk for development of diabetes and cardiovascular events. Further, we conclude that circulating death receptors in the plasma also can be associated to CVD-related apoptosis in SLE. Our findings provide clinical confirmation of the “response-to-injury” hypothesis of atherosclerosis that have been proposed based on findings in experimental models.

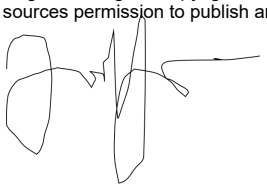
The second part adresses immune responses involved in the the mechanisms of atherosclerosis. Two different vaccine strategies targeting the peptide P45 of apolipoprotein B100, that consitutes the protein pat of LDL, were used study the inflammatory portion of atherosclerosis.The immunomodulatory vaccine formulations were shown to reduce atherosclerotic lesions in the subvalvular area and aortic arch in experimental studies. We also demonstrate that antibodies targeting this apoliprotein B 100 peptide sequence has a similar protective effect. The mechanisms responsible for these protective effects remain to be fully characterized but may include improved clearance of oxidized LDL and inhibition of inflammation.

Collectively, findings in this thesis provide evidence that death receptor-associated apoptosis can be linked to cardiovascular outcome in the general population and in patients with systemic autoimmunity. Further, these findings suggest that targeting immune responses against oxLDL with vaccination and antibody strategies could represent possible approach for prevention of the autoimmune responses in CVD.

Key words: atherosclerosis, apolipoprotein B-100, cardiovascular disease, autoimmunity, systemic lupus erythematosus, inflammation

Classification system and/or index terms (if any)

Supplementary bibliographical information		**Language** **English**
ISSN 1652-8220		**ISBN 978-91-7619-768-4**
Recipient’s notes	**Number of pages** 89	Price
	Security classification	

Signature  Date 2019-03-29

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Cover photo by Ingrid Yao Mattisson

Aquarelle painting of the low-density lipoprotein (LDL) and monoclonal antibodies targeting the ApoB100 protein enwrapping the LDL particle.

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Department of Clinical Sciences, Malmö
Experimental Cardiovascular Research

ISBN 978-91-7619-768-4
ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University
Lund 2019

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MADE IN SWEDEN

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Abbreviations

ACE	angiotensin converting enzyme
ACR-DI	American college of rheumatology damage index
ADCC	antibody-dependent cellular toxicity
AGE	advanced-glycation end product
APC	antigen presenting cell
ApoB100	apolipoprotein B 100
ApoE	apolipoprotein E
$ApoE^{-/-}$	apolipoprotein E deficiency
BCR	B cell receptor
Beta2GLP1	beta 2 glycoprotein 1
BMI	body mass index
Breg	regulatory B lymphocytes
B6	C57bl/6 strain
CANTOS	Canakinumab Anti-inflammatory Thrombosis Outcome Study
cFLIP	cellular FADD-like interleukin-1
CHF	congestive heart failure
CIRT	Cardiovascular Inflammation Reduction Trial
CTB	cholera toxin B subunit
CVD	cardiovascular disease
DAMP	danger-associated molecular pattern
DC	Dendritic cells
EAE	experimental autoimmune encephalimyelitis
Fab	antigen-binding fragment

Fc	fragment crystallization
FcR	fragment crystallization receptor
FADD	fas-associated protein with death domain
FoxP3	forkhead box p3
FasL	Fas ligand
GALT	gut-associated lymphoid tissue
GLACIER	Goal of oxidized LDL and activated macrophage inhibition by exposure to a recombinant antibody
HFD	high fat diet
hsCRP	high-sensitivity C reactive protein
HSP60	heat shock protein 60
ICOS	inducible T-cell costimulator
IDL	intermediate-density lipoprotein
Ig	immunoglobulin
iTreg	inducible Treg
IFN-γ	interferon γ
IL	interleukin
ILC	innate lymphoid cell
JUPITER	Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin
LDL	low-density lipoprotein
LDLR	low-density lipoprotein receptor
$LDLR^{-/-}$	low-density lipoprotein receptor deficiency
Lp(a)	lipoprotein a
MCP-1	monocyte chemoattractant-protein 1
MDA	malondialdehyde
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MI	myocardial infarction

nTreg	natural regulatory T lymphocyte
NK	Natural killer
NKT	natural killer T lymphocyte
OVA	ovalbumin
oxLDL	oxidized low-density lipoprotein
PBMC	peripheral blood mononuclear cell
PAMP	pathogen-associated molecular pattern
PMN	polymorphonuclear
PRR	pattern recognition receptor
P45	peptide 45 of apolipoprotein B
P210	peptide 210 of apolipoprotein B
RA	rheumatoid arthritis
sBP	systemic blood pressure
SLE	systemic lupus erythematosus
SLEDAI	systemic lupus erythematosus disease activity index
SLICC	systemic lupus international collaborating clinics
Tcyt	cytotoxic T lymphocyte
Tfh	follicular T helper lymphocyte
TGF	transforming growth factor
Th	T helper lymphocyte
TNF	tumour necrosis factor
TNFR-1	tumour necrosis factor receptor 1
TRAIL	TNF-related apoptosis-induced ligand
TRAILR-2	TNF-related apoptosis-induced ligand receptor 2
TRAILR-2	TNF-related apoptosis-induced ligand receptor 2
Treg	regulatory T lymphocyte
TCR	T cell receptor
TLR	toll-like receptor
T1D	type 1 diabetes

T2D	type 2 diabetes
VLDL	very low-density lipoprotein

Preface

Will it be possible to vaccinate against cardiovascular diseases in the future?

The fact that atherosclerosis is an inflammatory disease and most importantly, the root of many cardiovascular diseases, makes it tempting to imagine that a vaccine can protect against the underlying causes. The complexity of the disease has surprised researchers for many years. Even though the disease is mainly regarded as a Wester world disease where environmental factors are of importance, epidemiological studies suggest heritability partly plays a role too.

There is evidence that atherosclerosis has been around for centuries, where inflammatory plaques even have been detected in ancient Egyptian mummies with well-preserved intact vessels. The word atherosclerosis itself reveals the fact that the disease was observed in Europe as well. The direct translation from the Greek language can derive the word “atherosclerosis” as ‘athere’ meaning gruel accumulation, and ‘sclerosis’ meaning hardening.

Almost 30 years ago, researchers wanted to induce atherosclerosis in rabbits by vaccinating them with ‘bad cholesterol’, so-called low-density lipoprotein. Unexpectedly, the animals displayed a decreased amount of atherosclerosis in their vessels, why which they suspected the low-density lipoprotein administration gave rise to protection similar to the vaccination of infectious diseases. This experiment became the initial step to develop a protective vaccine against atherosclerosis.

With past research, a conclusion can be drawn; atherosclerosis is not a modern disease and was seen early on in several places worldwide. The mechanisms are of inflammatory nature where both genetic and environmental factors play a role. Still, there are mysteries that remain to be solved regarding atherosclerosis and the mechanisms of the disease. Hopefully, the present investigations in this thesis can add additional knowledge even though there is still extensive research ahead of us. With that said, the future of modulating the immunity in atherosclerosis is bright, exciting and can only be improved.

Aim

The overall aim of this thesis is to study immunological responses and autoimmunity in atherosclerosis.

Specific aims of this thesis include to identify the risk of developing atherosclerosis in individuals of the general population and individuals with autoimmune inflammation. Also, to investigate if circulating levels of inflammation-associated markers could be linked to cardiovascular disease-related events. This thesis also covers *in vivo* studies involving the use of vaccination strategies, with the rationale that individuals identified to be at risk of developing cardiovascular diseases would benefit from a therapy targeting the immune responses of the disease. The immune-modulating therapies examined in this thesis are based on atherosclerotic-related antigens with hopes to prevent and/or regress atherosclerosis in a systemic lupus erythematosus mouse model with lipid-driven inflammation. Finally, this thesis aims to study the molecular mechanism of active and passive immunization with apolipoprotein-derived antigens and antibodies to acquire suppressive protection.

The immune system

On a daily basis, our body is being exposed to viruses and bacteria. But because of the immune system, we rarely get sick. Its main tasks are the regulation of invading pathogens as well as the maintenance of tolerance against our own tissue. This is mediated by several defence strategies, taking place in parallel in a highly-intertwined way with the main goal to defend our body against pathogens. The first line of defence is made up of a physical barrier consisting of our skin, mucosal membranes and microbial flora. However, in some cases the pathogens might be able to penetrate the anatomical barriers or the microbial flora might be temporarily suppressed. Thus, we and other multicellular organisms possess additional protective mechanisms known as immunity, more specifically the innate and adaptive immune system, which requires both cell-mediated and humoral (cell-free) immunity.[1,2]

Innate immunity

The innate immune system comprises monocytes, macrophages, dendritic cells (DCs), polymorph nuclear (PMN) cells and natural killer (NK) cells. These are immediately encountering pathogens and recognize the foreign material by their so-called pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs). PAMPs are specifically expressed on pathogens with highly-conserved structures. Similarly, the PAMPs are detected by the highly-conserved pattern recognition receptors (PRRs) on our cells, such as toll-like receptors (TLRs) and scavenger receptors. If any of the innate immune cells interact with a PAMP, they start to alert surrounding cells by secreting cytokines, which are biologically active molecules attracting other immune cells.[3]

Macrophages and dendritic cells are antigen-presenting cells (APC's) that can direct their defence against the pathogen by displaying the foreign material to other inflammatory cells through the major histocompatibility complex (MHC) molecules.[3] Another important cell type is the natural killer T lymphocyte (NKT) cells, with properties to recognize a certain type of antigens. They display properties similar to NK cells and T cells, having a $\alpha\beta$ T cell receptor (TCR) but also express classical NK markers. Most importantly this cell type recognizes CD1d, a molecule

presenting glycolipids or other complex antigens. The best known NKT cells express an invariant αTCR chain and respond rapidly to inflammatory signals to exert important regulatory responses of the innate immune system. [4,5] Similar to the fast NKT cells, innate lymphoid cells (ILC's) are also able to respond immediately to contribute to protection. These cells reside mainly in the mucosal linings and have important roles in helminth infections or enteric pathogen invasion.[6]

Additional mediators of the innate immune system are various biologically active molecules, mostly proteins circulating in the blood system. During a pathogenic attack, the complement system and acute phase mediators bind to the pathogens. This action is called opsonisation and stimulates macrophages to eliminate the particle. Our body uses the same mechanism to dispose of internal debris thus, maintaining homeostasis.[7]

Adaptive immunity

Although the innate and the adaptive immune responses interrelate, there are differences distinguishing them in terms of actions. Compared to the fast-acting innate immune system with a lower degree of specificity, T cells and B lymphocytes of the adaptive immune system mediate enormously specific immune responses. These cells are produced in the bone marrow, maturate in the periphery after encountering antigens[8] and recognize antigens by their TCR and B cell receptor (BCR), respectively. As a result of the highly specified V(D)J that enables specific affinity for certain antigens, none of these receptors are alike the other one. For this reason, T and B cells have an immeasurable receptor variation and can mediate enormously specific response against invading material. Additionally, the adaptive immune cells have the property to acquire immunological memory for each of the specific antigens they encounter.[9,10]

Immunological responses

Antigen-presenting cells

APC's are the link between the innate and adaptive immune system. All APC's have the ability to internalize extracellular antigens and present the most immunogenic peptide through their MHC. There are two classes of MHC with the ability to present foreign molecules to remaining immune cells in the circulation. MHC I can be found on all nucleated cells and presents intracellular pathogen peptides. MHC II can only be found on APC's and express peptides from engulfed antigens but mediates a

strong and specific response against the antigen. This process is also an attractive target for immunomodulation since it directs T and B cells to the antigen.[11,12]

Costimulation

It requires two essential signals from the APC in order to activate a T cell. The first important signal is the ligation with the antigen-presenting MHC-complex. Secondly, ligation with a costimulatory molecule with the receptor present n the T ell is needed to elicit any further effector mechanisms. Costimulatory ligands are only present on fully activated APC's and also serves as a check-point of activation. Another set of molecules known as co-inhibitory molecules act reciprocally with the costimulatory molecules until the APC is properly activated. Examples of costimulatory receptors of the T cells are CD28, with belonging ligands CD80 and CD86 present on an APC. During non-pathological conditions, the MHC and costimulatory activation remains at a lower level. On the other hand, when innate immunity is activated it will upregulate co- stimulatory molecules and increase MHC II-antigen molecules to activate the adaptive immune system. This will break the homeostatic state of tolerance and initiate an immune response.[13-15]

T lymphocytes

The two major cell types of the adaptive immunity are T and B lymphocytes. Naïve T lymphocytes are exposed to the antigen through APC's either in lymphatic organs by circulating dendritic cells, or by macrophages at the sites of inflammation.[15] The exposure to an antigen turn the naïve T cells into a state of maturation and also decide their fate. There are two main subpopulations of the T cells that can be distinguished based upon their surface markers, CD4 and CD8. CD4 positive cells are known as T helper lymphocytes (Th) and CD8 positive cells are known as cytotoxic T lymphocytes (Tcyt).

Tcyt activation occurs by foreign peptide-antigens presented on mainly MHC class I molecules in combination with a costimulatory signal via the T cells CD28 molecule and CD80 or CD86 on the APC.[16] The cytolytic actions of the T cell are mediated through the cytotoxins perforin and granzym in order to eliminate unsuitable cells through a controlled cell death program, also known as apoptosis. A Tcyt expresses Fas Ligand (FasL) and will interact with the Fas receptor. The linkage between Fas and FasL enables perforin to form a pore-channel into the target cell and granzyme to activate an intracellular caspase cascade leading to cell death of both cells involved. This action enables clearance of inappropriate cells, for example, infected cells, self-reactive or cancer cells.[17-19]

The CD4 positive T helper cells mediate their actions by secreting cytokines to attract other immune cells to the site of inflammation for instance, B cells to initiate

their production of antibodies against the antigen. Th cells are both pro- and anti-inflammatory, given their high plasticity and number of subpopulations. Based on the environment and antigen, the naïve Th cell will be polarized to become either an effector or memory Th1, Th2, Th3, Th17, Tr1, Treg, Th9 or follicular Th (Tfh). Thus far, the most studied T helper cells are Th1, Th2, Th17 and regulatory T cells (Treg, Figure 1).[20]

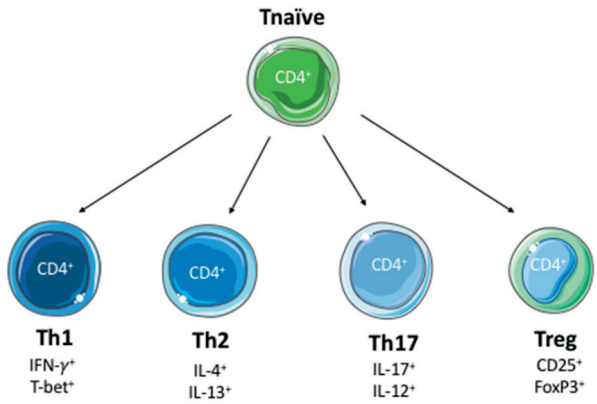

Figure 1. The most common T helper subsets A naïve CD4+ T cell can develop into several subtypes, depending on environment, activation cytokines and surrounding immune cells. The most abundant T helper subtypes are; T helper 1 (Th1), T helper 2 (Th2), T helper 17 (Th17) and regulatory T cells (Tregs). Illustration inspired by Grönberg et al, Stemme et al. and Szabo et al.[20-22]

The Th1 cells are an example of a pro-inflammatory Th subset. This subset can be distinguished based on their high expression of the transcription factor T-bet and interferon-γ (IFN-γ) secretion, which leads to macrophage recruitment and secretion of other pro-inflammatory cytokines to the site.[21] Amongst many diseases, Th1 cells are thought to drive the pathogenesis of atherosclerosis as indicated by studies showing a predominant IFN-γ secretion by atherosclerotic plaques.[23] This is further supported by a study demonstrating that 40-50% of all CD4 positive cells of aorta from atherosclerotic mice are indeed Th1 (defined as $CCR5^{+}T\text{-}bet^{+}FoxP3^{+}$).[24]

Th2 cells are defined as interleukin (IL) 4 secreting cells, a cytokine with the property to suppress Th1 polarization.[25] Alongside with IL-4, the Th2 cells secrete IL-13 as well. This cytokine has been shown to shift the macrophages from the inflammatory phenotype M1 to the anti-inflammatory phenotype M2.[26-28]

Another subtype of T helper cells is the more recently discovered Th17 cell. This subtype is suggested to be involved in several autoimmune diseases, having a pro-inflammatory effect.[29] After the discovery of the Th17 cells with a high production of interleukin-17 (IL-17), which is triggered by IL-12, there were several studies showing an increased population of the cell subset in autoimmune diseases such as

systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and type 1 diabetes (T1D).[30-32] Additionally, Th17 cells are involved in cardiovascular diseases where they for instance have a detrimental role in hypertension. [33,34] This rationale has stimulated new emerging therapies targeting the IL-17 pathways in both autoimmune and cardiovascular diseases[35-37]

Tregs are another subset of the T helper cells with immunosuppressive and antigen-specific properties. They perform suppression of pathogenic and self-reactive lymphocytes to maintain immunological tolerance. If the Tregs fail to restrain Th1 and Th17 immune responses, these proinflammatory T cell subsets might contribute to autoimmunity.[38] The natural Treg (nTregs) are differentiated in the thymus after encountering antigens. They are also of great importance in preserving homeostasis and prevent autoimmune reactions. Inducible Treg (iTregs) are in contrast differentiated in the periphery as a response to environmental cytokines such as interleukin-10 (IL-10) and transforming growth factor (TGF)-β, specific for iTreg.[20,39]

There are several subpopulations of Tregs but the most extensively studied one is denoted $CD4^+CD25^+FoxP3^+$. They differentiate in the thymus and can be found mainly in the periphery, distinguished by the expression of intracellular forkhead box P3 (FoxP3), a highly specific transcription factor required for maturation of the $CD4^+CD25^+FoxP3^+$ Tregs.[39,40] The importance of the Tregs suppressive actions have been identified in several immune relate diseases. For example, individuals with active SLE have deficient $CD4^+CD25^+$ Tregs with a lower expression of FoxP3 mRNA, supporting the hypothesisthat immunologic tolerance against self-antigens are lost.[41]

B lymphocytes

B lymphocytes are antibody producing cells, a property that associates them with the humoral immunity. However, B cells are also critical for the activation of cellular immunity by participating in T cell activation through antigen-presentation, co-stimulation and cytokine secretion. Once the immature (“transitional”) B cell leaves the bone marrow, membrane bound immunoglobulin (Ig) M and D are present on the cell membrane as part of the BCR complex. When the B cell has relocated into a lymphoid organ, the BCR will most likely encounter antigens presented by APC’s. This will differentiate the B cell to either a short-lived plasma cell or direct it to the germinal center of the lymphoid organ. Here, the B cell undergoes clonal expansion and class-switching. This will enable the B cell to produce high-affinity antibodies against the antigen, thus creating a highly specific memory B cell.[42] Depending on the location and environment of cytokines, B cells take on different tasks in the immune response.

In order to understand the enormous complexity of B lymphocytes, there are a B cell subset belonging to the innate immunity worth mentioning. Regardless of the adaptive immune system these B cells, known as B1 B cells, are known to be T cell independent, being able to elicit naturally occurring antibodies independently. They reside in the peritoneal cavity where they produce so called natural IgM antibodies directed against bacterial antigens such as highly conserved phospholipids and carbohydrate structures. These cells and their belonging antibodies are contributing to a constitutive protection through innate immune responses, which is thought to have evolved along with evolution.

The B cell portion that are mediators of the adaptive immunity and thus T-cell dependent are known as B2 B cells. The most dominant subset (>85%), consisting of follicular B cells and marginal zone B cells. They contribute to regulation of the adaptive immune responses as well as antigen-specific antibodies either as plasma cells serving as an antibody pool, or a memory B cell producing antigen-specific antibodies.[43] Follicular B cells reside in the spleen and lymph nodes, communicating with the T helper cells. Marginal zone B cells are short lived B cells residing in the outer part of the spleen, making it possible for them to capture antigens in the draining blood to present to T cells.[44,45]

Recent advances in the research of T cell-dependent B cells indicate that they are of proinflammatory nature. This is most clearly seen in chronic inflammatory states, where the B cells stimulate pathogenic T cells to secrete pro-inflammatory cytokines. An inflammatory disease with a pronounced B cell involvement is SLE. The central tolerance of B cells is altered because of a failure in the negative selection, giving rise to an abnormal BCR with higher affinity to self-material. This is supported by experimental studies where point mutations are introduced with an effect of altering a self-tolerant BCR into a self-reactive BCR.[46,47] This has been further supported by studies depleting all B cells, which results in an amelioration of inflammation.[48,49]

Besides promoting responses against antigens through antibody-specific protection, some subsets of B cells are thought to secrete IL-10 and thus, are of suppressive nature. This subset of B cells is called regulatory B cells (Bregs) with potential to dampen ongoing inflammatory activities from remaining immune cells, mostly T cells. Polarization to Breg occurs when the B cells are activated through CD40-mediated T cell co-stimulation but without a BCR-antigen interaction. Due to their role in the Treg/Th17 balance, to direct the B cell activation into a suppressive phenotype is an attractive approach for potential therapies.[49,50]

Immunoglobulins

As early as 1890, immunoglobulins and their actions were described by Behring and Kitasato, who initially observed that blood could neutralize bacterial toxins. This was later identified to be elicited by the cell-free serum, in which the protection could be transferred to other individuals. This was the starting point of the discovery of the humoral immunity.[51]

Immunoglobulins, or simply antibodies, are small Y-shaped glycoproteins present systemically with several biological properties such as neutralization of antigens, induction of phagocytosis, mediation of antibody-dependent cellular cytotoxicity (ADCC) and complement-mediated clearance.[52-54]

There are five isotypes of Ig's; IgG, IgM, IgA, IgD and IgE. Membrane-bound IgD and IgM antibodies build up the BCR on the naïve B cell, with the function to capture antigens for processing and presentation on the MHC complexes.[55] IgG is the most abundant immunoglobulin isotype and consists of four subclasses (IgG1, IgG2, IgG3 and IgG4). The IgG's play a central role in the immunological memory, which makes them an interesting candidate for immunomodulation.[56]

The antigen-binding fragment (Fab) determines the specificity of the antibody and allows for specific affinity maturation. The structure of the Fab is genetically determined in a complex somatic hypermutation and gene rearrangement process after encountering an antigen.[57] All biological effector functions exerted by antibodies are mediated by the fragment crystallization (Fc) region, found at the tail of the antibody (Figure 2). The Fc region of every antibody binds to its corresponding Fc-receptor (FcR), expressed by the APC's for further uptake into the cell. Depending on the antibody's structure it will bind to either the Fc-γ, Fc-α or Fc-ε receptor and elicit activating, neutralizing or inhibiting actions. Importantly, the inhibitory functions of FcR's are essential for tolerance, activation threshold and suppression of actions.[53]

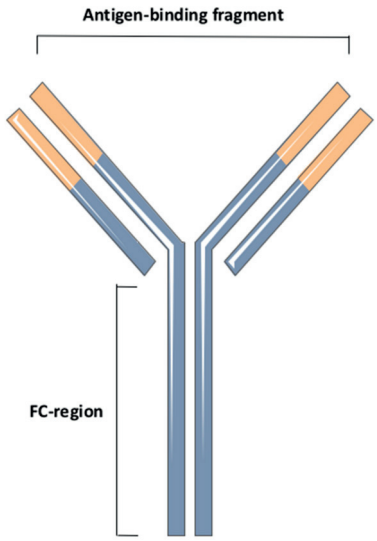

Figure 2 Schematic overview of an immunoglobulin G (IgG) molecule The antigen-binding fragment (Fab) of the antibody captures antigens. The Fc-region has the ability to bind to Fc receptors on effector cells to activate immune responses against the antigen. Illustration is a simplified version adapted from Schroeder and Cavacini.[57]

The biological mechanisms of antibodies make them excellent drug-candidates for anti-inflammatory therapy. Based on the Fc-FcR interaction, antibody-based drugs are available in the clinic to modulate adaptive immune responses, thus offering precise medicine with significant therapeutic benefits.[2,53,58]

Tolerance

Tolerance is defined as a state of unresponsiveness to an antigen originating from the own body of no harm. The adaptive immunity generates a highly specific protection and these mechanisms are tightly regulated by several tolerance mechanisms to maintain the adaptive immunity at a reasonable level of activation.[59]

Central tolerance is a crucial process occurring in the lymphoid organs where lymphocytes mature and acquire self-tolerance. T cells with a low affinity for the MHC-peptide complex expressed on APC's will not receive growth signals for survival and consequently die through apoptosis. This event is the first step of the maturation and is called positive selection, in order to make sure of the TCR capability to interact with APC's. The second step is clearance of T cells whose TCR react with MHC-self peptide complexes and is called negative selection. This step is somewhat stricter, most of the lymphocytes do not pass. However, a small

fraction of the lymphocytes does not interact with the antigens and will successfully be released into the periphery.

Following the central tolerance selection, there is also the peripheral tolerance selection. Because some autoantigens are only present in the circulation, there is an additional mechanism to avoid reactivity against these kinds of self-antigens. For this reason, several protective mechanisms will occur when a T cell interacts with self-antigens in the periphery. Those protective actions include T cell anergy (a state of unresponsiveness after activation), cell cycle arrest or death receptor activated-induced cell death, making sure that all lymphocytes are directed against foreign antigens. [60,61]

However, when the T cell finds a correct foreign antigen they need to be costimulated too, in order to differentiate and become effector cells. The costimulatory molecules are regulated by PAMP's or DAMP's of the innate immune system. Therefore naïve T cells, who haven't encountered an antigen, are tolerant against self-antigens due to the lack of costimulation.[59] When the self-tolerance fails for various reasons, it is said to be in a state of autoimmunity. This can happen both systemically and locally.

Apoptosis

Apoptosis, also known as programmed cell death, is a complex regulatory process of controlled clearance of dying cells to maintain homeostasis and vitality in the body. Moreover, it is a cellular defence mechanism to resolve inflammation or DNA damage. It is also a way to eliminate cells in the tolerance process.[62] Apoptosis was discovered as early as 1858 by Virchow who described it as a biological mechanism to deal with gradual decay and bring death to living parts that was worn out.[63] In 1972, apoptosis was fully characterized by Kerr, Wyllie and Currie, who also later came to develop morphological criteria to discriminate apoptosis from the sudden uncontrolled cell death due to physical traumas, known as necrosis.[64,65]

A cell is considered to have undergone apoptosis when the cell performs an intracellular defragmentation process through an enzymatic cascade before the cell membrane is dissolved. This controlled action avoids leakage of the cytosolic and nucleic materials into the system, which otherwise could be recognized by the immune system as an injury and consequently activate immune responses.[66]

Apoptosis can be initiated through two pathways depending on environmental and physical factors; the extrinsic and the intrinsic apoptosis pathways. Both of the pathways will eventually lead to activation of caspases, a family of cysteine endoproteases with the ability to hydrolyse peptide bonds in a catalytic reaction

depending on cysteine residues. The intrinsic pathway is triggered by cellular stress and DNA damages where apoptotic factors are released from the mitochondria to create the common end product of active caspase 3.[67,68]

Death receptor-activated apoptosis

The extrinsic pathway of apoptosis is activated through death receptors, which are transmembrane proteins belonging to the TNF receptor superfamily. These include the tumor necrosis factor (TNF) receptor 1 (TNFR1), TNF-related apoptosis-induced ligand receptor 1(TRAILR1) and 2 (TRAILR2) and Fas/CD95.[67,69] Common for all death receptors are the cytosolic domain termed the "death domain". When soluble ligands bind to the extracellular portion of the receptors, this leads to trimerization of the cytosolic death domain and forms the death-inducing signalling complex (DISC) to convert the downstream inactive procaspase 8 into active caspase 8. Further, caspase 8 now has two prospective ways of action; it can either continue to proteolytically cleave the downstream caspases 3, 6 and 7 of the extrinsic pathway and complete apoptosis, or it can trigger the inhibiting molecule cFLIP (cellular FADD-like interleukin-1 converting enzyme inhibitory protein) to prolong survival of the cell. Caspase 8 needs to convert procaspase 3 into its primary apoptotic mediator caspase 3 to carry out apoptosis completely (Figure 3). This is the driving signal of DNA fragmenting, the final step in the apoptosis.[70,71]

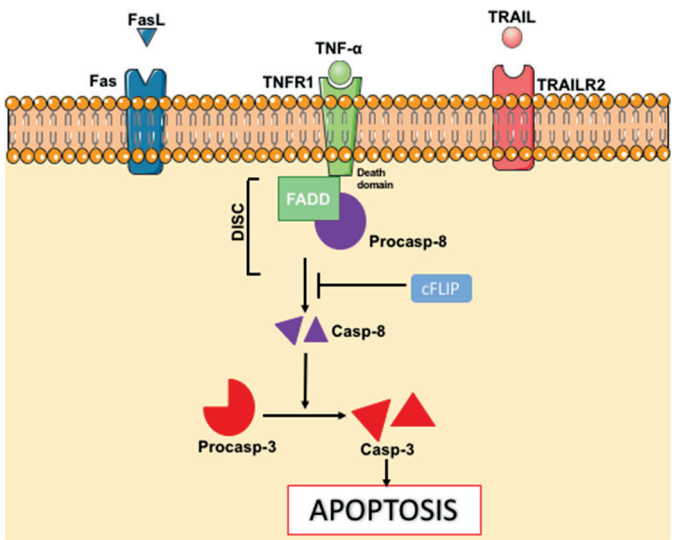

Figure 3. Extrinsic apoptosis pathway Apoptosis is triggered by a death receptor ligand binding to its receptor. The death domain transmits intracellular signaling to dimerize with FADD. The DISC complex is formed when procaspase-8 is recruited. If cFLIP doesn't inhibit the process, caspase-8 will be formed which cleaves procaspase-3 into the active mediator caspare-3. Illustration is adapted from McIlwain, Berger and Mak.[70]

Soluble death receptors

The death receptors can be detected in a soluble form after activation. The interaction of the ligand with its specific death receptor initiates several signal transduction pathways such as apoptosis described above. Furthermore, it is also proteolytically cleaved from the cell membrane in a process known as ectodomain shedding. This phenomenon is still under investigation, but is suggested to have an opposing action of the innate immune responses.[72,73] The soluble death receptor can thus be involved in negative feedback of the ongoing inflammation when ligating with its pro-inflammatory ligand This mechanism has most likely evolved to establish a balance between inflammation and tolerance.[74] Elevated levels of circulating death receptors can therefore reflect the ongoing inflammatory responses. For example, TNFR1 is associated with myocardial infarction and CVD-related mortality. [74,75]

Cardiovascular disease

CVD are a group of diseases that affect the circulatory and vascular systems. Today, CVD-complications are the leading cause of mortality worldwide accounting for 30% of all deaths. Myocardial infarction (MI) and stroke are the most common (80%) causes of CVD mortality.[76] Most of the CVD-related events are a consequence of atherosclerosis, an inflammatory disease where lipids are deposited in the vessel walls. Today, a progressive increase in CVD prevalence has emerged. There are drugs targeting the symptoms caused by CVD, and also drugs used to maintain blood lipids at a healthy level. Most importantly, a change in lifestyle is the most beneficial strategy to prevent disease development and/or progression. [77] Although there are numerous approaches to prevent and control CVD related symptoms, the fact that atherosclerosis remain the cause of a majority of clinical cardiovascular events proves the need for additional therapies.[78,79]

Atherosclerosis

Atherosclerosis is a chronic inflammatory disease giving rise to major CVD complications worldwide with clinical manifestations such as stroke and myocardial infarction. The disease was first defined by Nikolai Anitschkow in St Petersburg year 1914. The word atherosclerosis can be translated from the Greek language derived as ‘athere’ meaning gruel accumulation, and ‘sclerosis’ meaning hardening As a silent disease that progresses unnoticed until a clinical cardiovascular event occurs, it has now emerged as a lifestyle disease.[80,81]

The atherosclerotic process is initiated when cholesterol-rich low-density lipoproteins (LDL) particles are entrapped in the vascular wall and modified by enzymes or reactive oxygen species forming a pathogenic oxidized version of LDL (oxLDL). The atherosclerotic lesions build up in all individuals over time, a process that is initiated as early as in the fetal stage of development. Whether the arterial plaques become benign or pathological by nature, it depends on both genetic and environmental factors, such as family history and diet respectively.[78,82]

The most common complication due to atherosclerosis is plaque rupture of the lesion causing thrombotic vessel occlusion. This can lead to fatal events such as

myocardial infarction and stroke, which together represents the most common causes of death worldwide. Depending on the location of the ruptured plaque, this will determine the clinical consequence. For instance, if a plaque ruptures in the coronary arteries it causes myocardial infarction. Conversely, if it is located in the carotid or cerebral artery, which supplies the brain with oxygenated blood, it is called a stroke. In order to resolve the acute clinical manifestations, these events are the primary target to treat with the drugs available today. [83]

Pathogenesis

Every organism is in need of cholesterol to maintain the cell membrane structure amongst other things. Cholesterol itself is not soluble in the blood and needs LDL and the apolipo B 100 protein (ApoB100) in the circulation.[84]

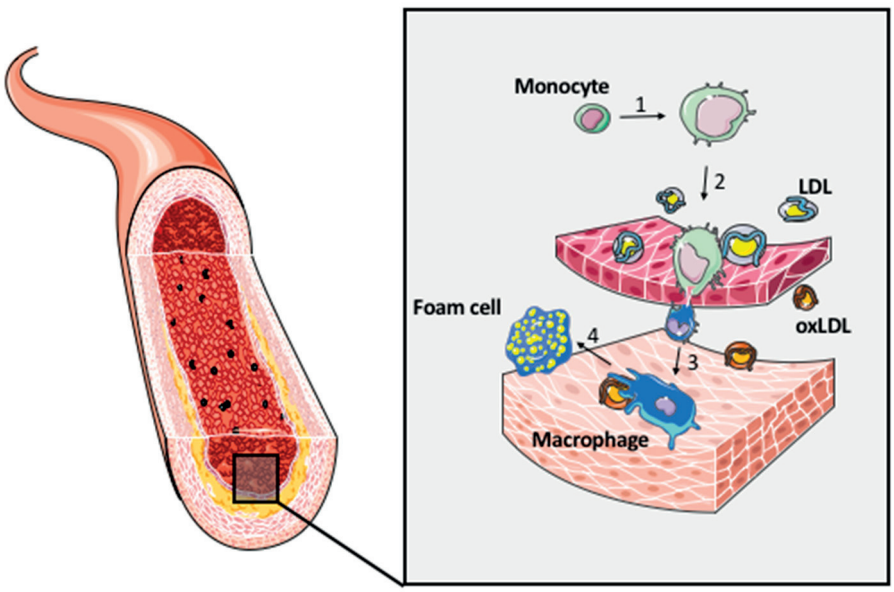

Figure 4. Foam cell formation in the atherosclerotic vessel Low density lipoproteins are entrapped in the intimal part of the vessel, get modified by environmental enzymes and reactive oxygen species. 1) This will activate endothelial cell and recruit monocytes which 2) migrate into the intima and differentiates to macrophages. 3) The macrophages develop to foam cells when they ingest oxLDL and 4) store it as lipid droplets intracellularly. Eventually, the foam cells will be eliminated by surrounding cells or accumulate as a necrotic core in the plaque. Illustration is inspired by Hansson and Hermansson.[89]

Atherosclerotic plaques contain varying amounts of LDL entrapped in the arterial wall. A key mechanism of the plaque development is oxidation of the LDL particle and accumulation of lipid loaded macrophages, referred to as foam cells (Figure 4).[78,85,86] Eventually, when LDL particles are retained in the vascular intima, they will be subjected to modifications by ions, reactive oxygen species and enzymes. These kinds of changes alter the composition of the LDL particle to oxLDL, strongly pro-atherogenic and also recognized by the PRR's as a foreign antigen.[87,88].

The PRR's are located on antigen-presenting cells and this will initiate an infiltration of monocytes, an important initiator of atherosclerosis. The monocytes differentiate into scavenger receptor-expressing macrophages as a response to oxLDL and the cells will ingest the oxLDL particles and store them intracellularly. Additionally, they will present peptides derived from oxLDL to T cells.[59,89]

Response to injury hypothesis

In 1976, Ross and Glomset postulated the response to injury hypothesis in the New England Journal of Medicine. They proposed two major events as drivers of lesion formation. This is initiated with a process occurring in all individuals at varying degree where endothelial injury leads to local inflammation. If this event is occasional, the injury will regress and leave a minimally thickened intima. However, if the injury is chronic, the repeated event to the arterial wall will have a higher rate of inflammation in relation to resolution. This will contribute to a more susceptible environment of the tissue where intracellular and extracellular lipids easily can accumulate. [90]

With this knowledge, it is now widely recognised that vascular injury is one of the drivers in the atherogenesis, including metabolic stress and oxLDL-mediated injury of the vasculature occurs.[91,92] The immunological defence in response to oxLDL injury includes a natural antibody response, macrophage activation that facilitates the removal and induction of adaptive T cells.[93,94]

Most research of immunity in atherosclerosis has focused on the myeloid cell contribution to atherosclerosis through foam cell formation and endothelial injury, but less is known about the T and B cells. While the role for macrophages in atherogenesis is established to be a major player in the development, the role for the adaptive cells seem to have multiple roles and work reciprocally with eachother.[95] Therefore, the role for the adaptive cells in atherosclerosis needs to be identified thoroughly, in order to develop better therapies.

Adaptive immunity in atherogenesis

Adaptive immune mechanisms are activated in response to atherogenic antigens in the plaque. Examples of these are modified LDL, beta 2 glycoprotein 1 (Beta2GLP1), heat shock proteins, lipoprotein a (Lp(a)) and matrix proteins modified by what is known as advanced-glycation end (AGE) products. Several autoantigens in atherosclerosis have been studied (Table 1).[59,96]

T cells comprise around 10% of all cells in the human atherosclerotic plaques. Of those, seventy percent are $CD4^+$ cells, and the remaining are $CD8^+$ cells.[97] T cells are present at all stages of plaque development (unlike the B cells, which are sporadically detected but instead might be present in the adventitial layer forming tertiary lymphoid organs).[98]

The adaptive mechanisms that have come to fascinate the field more and more, with new research emerging about the immunological responses during atherosclerosis. For example, one research group concluded that lymphocyte depletion resulted in a markedly reduced plaque size in a hypercholesterolemic mouse model, highlighting the importance of the T cells in the pathogenesis.[99] Another study could confirm these results by transferring $CD4^+$ cells to a similar model, where the lesion size increased by 164%, where an increased level of proinflammatory cytokines were detected.[100] Collectively, these data show that $CD4^+$ deficiency is protective while transfer of $CD4^+$ cells accelerates the disease. This made it interesting to further study the roles of the various $CD4^+$ subtypes Th1, Th2, Th17 and Tregs to establish the T helpers' role in atherosclerosis.

Table 1. Antigens involved in the atherogenesis Adapted from Milioti et al.[96]

Autoantigen	Function	Generated by	Reference
Oxidized low-density lipoprotein (oxLDL)	Cholesterol carrier protein	Enzymatical modifications of LDL	[101]
Oxidized phospholipids (oxPL)	Cell membrane constituent	Oxidative stress of phospholipids	[102]
Beta2 glycoprotein 1 (Beta2GLP1)	Circulatory thrombin inhibitor	Proteolytic cleavage in coagulation pathway	[103]
Lipoprotein a (Lp(a))	Repair mechanisms	Hepatocytes	[104]
Lipoprotein lipase (LPL)	Lipid hydrolyzing enzyme	Intestinal cells	[105]
Advanced glycation-end products (AGE)	Harmful protein/fat molecules combined with sugar	Glycation of soluble protein/fat molecules	[106]
Heat shock proteins	Promote degradation after stress/injury	Nucleus	[107,108]

Th1 cells in atherosclerosis

Th1 cells express the transcription factor T-bet as compared with the increased IL-4 and IFN-γ secretion. When depleting any of these factors, or mediators affecting expression of these, results show that the atherosclerotic development is decreased.[109-112] Further, the Th1 subtype $CD4^{+}CD28^{null}$ confirms the proatherogenic properties by secreting high levels of IFN-gamma and IL-12. This cell type is however only present in humans, which is why depletion and transfer studies with this population are not possible to conduct in a mouse model. [113,114]

Th2 cells in atherosclerosis

It is still unclear whether the Th2 cells are pro or anti-inflammatory in atherogenesis Secretion of IL-4, IL-5 and IL-13 is one of the major characteristic of the Th2 subtype. The introduction of IL-5 secretion stimulates B1 cells to produce protective natural IgM, which is seen in one study where atherosclerosis is attenuated.[115] This is also why the research regarding Th2 cells has focused on depletion of these cytokines to elucidate the function of the Th2 subset. In one study, depletion of IL-5 and IL-13 resulted in aggravated atherosclerosis development. On the other hand, hypercholesterolemic Apolipoprotein E deficient ($ApoE^{-/-}$) mice with their associated low levels of IL-4 developed smaller plaques. [28,115,116] Moreover, high levels of IL-4, IL-5 and IL-13 are associated with the innate type 2 ILC's, a population with a protective role in atherosclerosis.[117-120] Although results are inconsistent regarding the role of Th2 in atherosclerosis, a conclusion can be drawn that the cell subset are involved in a protective response, including promoting B cells to produce natural antigens.

Th17 cells in atherosclerosis

The Th17 T cell subset has been extensively studied in autoimmune states, indicated with findings supporting a role inflammatory diseases. In atherosclerosis Th17 cells are considered to be proatherogenic, because of the proinflammatory responses induced in response to oxLDL.[121] In line with these results, inhibiting or blocking IL-17A *in vivo* resulted in reduced atherosclerosis.[122,123] However, some sources indicate that this population has an anti-inflammatory profile by showing that the lack of IL-17 accelerates atherosclerosis in a hypercholesterolemic mouse model, which could be resolved by administering IL-17.[124]

Regulatory T cells in atherosclerosis

An important regulator of autoimmune responses is Tregs, with a crucial role in suppression of autoreactive T cells.[125] Tregs have been proposed to ameliorate the inflammatory environment in atherosclerosis lesions, as shown by transfer experiments with $CD4^{+}CD25^{-}$ Tregs. In line with this, atherosclerosis is attenuated by IL-10 deficiency, an anti-inflammatory cytokine produced by Tregs.[126,127] This

highlights the protective function of the Tregs in atherosclerosis with opportunities to explore this mechanism in specific therapies targeting the inflammatory Th1 cells.

B cells in atherosclerosis

The B cells are less extensively studied in atherosclerosis, but are also contributing to the pathogenesis. Mostly regarded as protective, the B1 B cells have an important role in the protection, where the natural antibodies specifically target oxLDL and apoptotic cells. Furthermore, treatment with a neutralizing antibody targeting CD20 on B2 cells resulted in attenuated development of atherosclerosis in $ApoE^{-/-}$ and LDL receptor deficient ($LDLR^{-/-}$)mice, which strengthens the protective role of B1 B cells. [128-130]

Type 2 diabetes

Type 2 diabetes (T2D) is a metabolic disorder and major risk factor for CVD resulting in chronic elevated blood glucose, so-called hyperglycemia. This affects the insulin-producing β-cell islets of the pancreas.[131] The disease is characterized by insulin resistance and/or dysregulated insulin secretion. At an early stage of the disease, the patient has exaggerated levels of insulin to respond to the elevated blood glucose levels. Eventually in the later stages of the disease, the insulin production declines due to β-cell failure.[132,133]

T2D is a multifaceted disease caused by a combination of genetic and environmental factors which together will result in a disrupted glucose homeostasis. Classical risk factors for T2D include age, gender, obesity, smoking, ethnicity, T2D family history, physical activity level and gestational diabetes mellitus. [134] In the context of cardiovascular disease complications, patients suffering from T2D have a 2-3 fold increased risk of experiencing a CVD related complication.[135]

It is implied that elevated levels of pro-inflammatory cytokines can be associated with T2D and its complications. Supporting this, studies show that pro-inflammatory cytokines trigger dedifferentiation of β cells.[136,137] Similar to atherosclerosis, this suggests that general inflammation has a role in the development of the disease. Also, the activity of the apoptotic marker caspase-1 can be linked to high glucose, suggesting that apoptosis plays a part in diabetes and its complications.[138,139]

Cardiovascular disease in type 2 diabetes

An extensive meta-analysis from 2007 to 2017 reports the prevalence of CVD as approximately 50.3% amongst persons with T2D.[140] Risk factors for CVD in T2D include hyperglycemia, hypertension, dyslipidemia, obesity, lack of physical activity and smoking. Control of the hyperglycemia is the first intervention in T2D to lower the symptoms. In most cases, this treatment is enough in combination with a better lifestyle and diet. However, the role of ongoing low-grade inflammation has recently been focused on for these patients.[141]

Metabolic abnormalities such as insulin resistance contributes to elevated environmental stress in T2D. Endothelial dysfunction, thickening of the carotid intima -media and myocardial ischemia can also frequently be seen. These data highlight the increased risk of CVD in T2D, but the biggest challenge is to identify and prevent CVD-related events in T2D patients before they occur. The fact that cellular and metabolic stress underlie not only atherosclerosis but also T2D too, is an interesting aspect in terms of patients at risk. There are today not any standardized procedures implemented in the clinic to identify and assess the degree of atherosclerosis in these patients, which could help to prevent future cardiovascular events.[142]

Research has moved forward and there are now studies that have been published with suggestions of how to target the CVD-related inflammation in T2D patients. In fact, the atherosclerotic burden was measured as carotid intima-media thickness (cIMT) and ankle-brachial pressure index in one study and this could be independently associated with clinical manifested CVD in T2D individuals. The measurement of atherosclerotic burden with these methods can serve as a valid surrogate marker to identify CVD earlier with the rationale to enable preventive treatments.[142] The increased CVD burden can also be linked to LDL oxidation in T2D individuals. For example, one observation suggests that autoantibodies against lipoproteins may act as biomarkers for both micro and macro vascular complications in diabetes.[143] Indeed, if the lack of robust biomarkers and immediate assessment of CVD-related inflammation can be resolved, there is a good chance to complement the T2D treatment with an immunomodulatory medication to lower the prevalence of CVD in this patient group.

Autoimmunity

Autoimmunity is a condition in which the immune system mediates a pathological response against its own healthy tissue, seen as self-antigen. The tolerance for self-antigens is controlled by multiple mechanisms but some self-reactivity is considered to be a price needed to pay for maintaining effective defence against all types of pathogens. It has become apparent that these mechanisms of tolerance are sometimes dysfunctional during maturation of immune cells, leading to an impaired capacity to distinguish between pathogens and self-antigens. Inevitably this gives rise to autoimmune diseases of varying kinds, depending on the targeted area. Thus, autoimmunity displays huge heterogeneity in disease phenotype and its clinical manifestations.[144] Autoimmune diseases can be both systemic (systemic lupus erythematosus, autoimmune thyroiditis, rheumatoid arthritis) and organ-specific (type 1 diabetes, psoriasis, ulcerative colitis).

Loss of tolerance

Autoimmunity arises when the immune system fails to sustain tolerance against self-antigens. Hence, the tolerance is lost and the immune system is then allowed to react to antigens it has previously ignored. Over the past sixty to seventy years, the concept of autoimmunity has been explored to understand the immunological processes. The 'self-non-self' theory emerged around 1950, with the explanation that responses directed against 'self' antigens took place because the immune system confused true foreign antigens and structurally similar 'self' antigens. The model that postulated an altered recognition of antigens lead to the Nobel Prize in physiology and medicine in 1960.[145]

Autoimmune mechanisms are involved in several diseases. Because of the shared aetiology some individuals can develop multiple autoimmune diseases. It is not unusual that some individuals are diagnosed with "overlapping syndrome" due to the resembling clinical features although different pathologies. This is clinically challenging in finding the most suitable treatment approach. This is exemplified by RA and SLE patients who often exhibit premature atherosclerosis, most likely due to the ongoing systemic inflammation.[146,147] If we had a better understanding mechanistically of how exactly inflammation contribute to atherosclerosis,

therapeutic strategies targeting both inflammatory mechanisms would be an ideal choice instead of treating the diseases as two separate conditions.

One important theory about why the prevalence of autoimmune diseases have increased, is the explanation that enhanced hygiene and better pathogen-reducing therapies have reduced our exposure to microbes. In turn, this permits the immune system to lower its activation threshold and better react to 'self' antigens.[148]

Mechanistically, autoimmunity arises when a failure in the central or peripheral tolerance maturation of lymphocytes occurs. To prevent autoimmunity, the body naturally excludes autoreactive cells through deletion, anergy or suppression. The breakdown of any of these processes gives rise to autoreactive lymphocytes and depending on 'how, when, where, why' the failure occurred it will also determine which autoimmune disease results. [149] One example of loss of tolerance is when B cells become activated to produce antibodies against self-antigens. This can be a result of incorrect somatic hyper-mutation of the BCR to bind to improper self-antigens presented on the APC's MHC such as nuclear DNA particles, thus causing improper production of antibodies against nuclear material. [150]

oxLDL autoimmunity

Autoimmunity can develop when a self-antigen resembles a foreign antigen. This is one reason why oxidized LDL is highly pro-immunogenic, since the molecule resembles an apoptotic cell with its oxidative-specific epitopes.[151,152]

Björkbacka et al. described atherosclerosis as *"probably not an autoimmune disease in the classical sense but rather a state of local immune dysfunction resulting in an imbalance between naturally occurring autoimmunity and the regulatory immune cells that should control this autoimmunity"*[153] Indeed, atherosclerosis is not considered to be a classical autoimmune disease but an entity with several similarities to the pathological mechanisms. Antibodies against oxLDL have been extensively studied, especially the presence of the natural oxLDL-specific IgG and IgM antibodies. OxLDL-antibodies can be extracted from human and mouse atherosclerotic lesions, and the level of circulating plasma antibodies can be detected in both human and mouse. [154,155] However, epidemiological data of the association between plasma levels of anti-oxLDL autoantibodies and CVD are inconsistent with some studies reporting a correlation with severe atherosclerosis, while others suggest inverse associations.[156,157] Although very inconsistent, a pattern can be seen suggesting that IgM is protective and IgG is detrimental. This inconsistency has been evaluated in a CD40L deficient (defective in the CD40-CD40L costimulation) ApoE$^{-/-}$ model, which are also deficient in IgG. Data from the study suggest that the severity

of the atherosclerotic plaques is not caused by the specific isotypes of antibodies against oxLDL.[158]

Circulating antibodies against oxLDL

Antibodies against the LDL protein ApoB100 and its peptides have been identified, where a set of peptides emerged as highly atherogenic (Table 2). Passive immunization with antibodies targeting a specific antigen is not a new approach in vaccine development. Hence, this could be an attractive method to effectively modulate the immune responses in atherosclerosis.

Indeed, there are several studies that can link ApoB100 antibodies to CVD events. One study presents that lower levels of IgG and IgM antibodies targeting native and malondialdehyde (MDA) -modified peptide number 45 (P45) and peptide number 210 (P210) of ApoB100 can be associated with increased risk of coronary events.[159] Additionally ischemic patients with higher plasma levels of native P45-IgG could be associated with increased stabilizing collagen and smooth muscle cell growth factors present in the plaque and decreased pro-inflammatory cytokines. These results suggest an anti-inflammatory property of the antibody, which also has been seen to be correlated with decreased carotid stenosis and cardiovascular events.[160,161]

Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is an autoimmune disease with dysfunctional tolerance of the immune system against own healthy tissue in several organs. Characteristics of the pathogenesis of SLE comprises the production of autoantibodies directed against nuclear material together with organ damage and degradation of tissue. An important hallmark of SLE is the ongoing chronic inflammation, comparable with atherosclerosis. The pathological traits do however arise from a different set of mechanisms including B and T cell abnormalities, auto-antibody production and complement activation amongst many other pathological features. The production of specific antibodies directed against nuclear material present in the circulation is especially characteristic for the disease, where the autoantibodies form immune complexes in draining organs such as kidney and lymph nodes. This has damaging effects to the tissue and often causes inflammation in the organ.[162]

CVD in systemic lupus erythematosus

The incidence of CVD is significantly increased in SLE patients suggesting that the enhanced systemic inflammation contributes to a dysfunctional handling of oxLDL and other atherogenic antigens. For example, females with SLE have up to 50-fold increased risk of developing MI, which highlights the need for alternative preventive therapies to inhibit atherosclerosis in SLE.[147,163]

The mortality of patients suffering from SLE has decreased dramatically. The fact that the five-year survival rate has gone from 50% in the 1950s to 95% in the 2000s emphasizes the benefits of current treatments with corticosteroid and immunosuppressive drugs as the main pillars. As a consequence of the increased life expectancies, CVD's have emerged as the major cause of mortality amongst these patients.[164,165]

Unfortunately, there are unmet medical needs for SLE patients suffering from premature CVD and one major consequence of the difficulties to identify patients at risk. [166,167] Traditional CV risk factors (defined by the Framingham risk factor assessment[168]) do not fully identify SLE individuals at risk of CVD. [169] In one study, the authors could however identify an increased risk of stroke and coronary heart disease in younger SLE patients when assessing the Framingham risk score in a British cohort, but failed to find any causal relationships.[169,170] The fact that the incidence of CVD is significantly increased in SLE patients suggest that the enhanced systemic inflammation contributes to the dysfunctional protection against oxLDL and other atherogenic antigens. [171] In fact, serum from lupus patients containing immune complexes and activating cytokines have pro-inflammatory effects and can induce cholesterol accumulation in cultured smooth muscle cells. This happens due to the LDL-containing immune complexes present in the serum, which also highlights the fact that these individuals have a lower oxidation threshold against plasma LDL in general.[172,173]

Another contributing factor for inflammation in SLE is the impaired regulation of apoptosis. It has been demonstrated that soluble death receptors are systemically increased in SLE patients with cardiovascular complications, thus suggesting an intensified signalling through death receptor-activated organ damage that contributes to the cardiovascular inflammation and elevated apoptosis in SLE.[174]

CVD-related autoantibodies

In SLE, antibodies against oxLDL have been shown to cross-react with both anti-cardiolipids and beta2GP1 that further facilitate immune complex formation in SLE individuals.[175,176] Interestingly, the abundancy of IgG and IgM autoantibodies P45 and P210 are decreased in SLE individuals. In addition to this, those SLE individuals with CVD have an even lower level of these antibodies. This unexpected

finding suggests that there is an impaired antibody-mediated removal of LDL, as SLE individuals normally have excessive autoantibody production.[177]

Immunomodulating therapies targeting CVD

As a result of invaluable research throughout the years, medications such as statins, beta-blocker and angiotensin-converting enzyme (ACE) inhibitors amongst many others are now successfully implemented in the clinic as standardized protocols for CVD management. But as current treatments improve, new features of the disease arise. It is a fact that atherosclerosis is an inflammatory disease, but there are no therapies specifically targeting this portion of the disease.

Almost 30 years ago, the first experiments to target the inflammation induced by autoimmunity in atherosclerosis were performed, with the hopes to reduce the disease development. Oxidized LDL were administered to rabbits to confirm the hypothesis but instead of aggravated disease, the animals displayed decreased plaque formation compared to the controls. This unexpected finding was similar to the protective effect of immunization against infectious diseases and came to lay the grounds for further research of a vaccine targeting oxLDL.[83,178,179]

The mechanisms behind the idea of vaccination is to initiate the innate immune system to activate the APC's in order to induce an adaptive immune response and produce antibodies against the pathogen.[180] Additionally, adjuvants are a necessary component in a vaccine, and comprises a molecule that helps to activate the immune system[181,182] If immunomodulatory therapies targeting the immunological mechanisms of atherosclerosis succeeds to be generated, this will enable an improved outcome of CVD.

Modulating immune responses in atherosclerosis

Even with the success of lipid lowering medications, there are still individuals who are unresponsive to these therapies, thus revealing the need to target the inflammatory aspect of atherosclerosis. The JUPITER study (Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin) revealed a beneficial role for statin treatment and CVD events.[183] Despite this, statin treatment was not able to reduce other atherogenic particles such as very low-density

lipoproteins (VLDL) and intermediate-density lipoproteins (IDL).[184] Extensive research suggest that peptides of ApoB100, which is found in all lipoproteins, are an excellent target to direct immunomodulatory approaches to.[185] The fact that P45 antibodies are associated with a risk of suffering a CVD-related event supports the idea of an antibody-inducing vaccine directed against immunogenic peptides of ApoB100, such as P45.[186] The initial experiment of vaccinations against atherosclerosis was performed almost 30 years ago. By vaccinating rodents with LDL or oxLDL, it was shown that these animals were protected and developed smaller atherosclerotic lesions.[187-189]

Peptide-based vaccines

The fact that immunization with LDL was atheroprotective led to research focusing on the ApoB100 protein. Epitopes from ApoB100 were shown to be highly atherogenic, thus several of those peptides were evaluated together with adjuvants, administration route and preparation.

To identify certain especially interesting epitopes, a polypeptide library covering the sequence of apoB100 was created with synthesized peptides. This made it possible to recognize which peptides that attracted the most antibodies. Two especially interesting peptides were p45 (amino acid 661-680) and p210 (amino acid 3135-3136).[190] The highly immunogenicity of native ApoB100 peptides p45 and p210 were then used in vaccination experiments to examining the immune responses. Strikingly, the immunization was shown to mediate protective immune responses, as seen by a reduced atherosclerotic plaque amount in aorta and the subvalvular region in experimental studies.[191]

Oral tolerance induction with ApoB100 peptides

Oral tolerance is defined as suppressive immune responses to antigens given by oral rout of administration. The mucosal barrier in the intestines work in concert with lymphoid cells to promote a defence against foreign pathogens. In addition, an adjuvant is needed to promote presentation to immune cells.[182] Cholera toxin B subunit (CTB), with its high membrane-binding capacity, is used as an adjuvant.[192] Mechanistically, CTB mediates binding to the gut cells and presentation of the antigen to APCs. This induces antigen-specific T cells that strongly suppress antigen-activated effector T cells.[193] The suppressive effects are believed to act through enhanced IL-10 and TGF-β production that also can be associated to an increased gene expression of IL-10 and TGF-β mRNA.[193]

CTB-coupled antigens have been evaluated for immunomodulation in immunological diseases such as allergy, diabetes and experimental autoimmune encephalomyelitis (EAE).[182,194] Based on the strong evidence of suppressing

effector T cells in the use of CTB-coupled atherogenic antigens have been assessed as an oral vaccination to induce peripheral tolerance against LDL. The use of an intranasal vaccination with P210-CTB decreased atherosclerotic lesions with 35% in $ApoE^{-/-}$ mice, accompanied by increased Tregs with suppressive effects on ApoB-100 re-challenge *in vitro*. Additionally, IL-10^{+} T helper cells and IL-10 mRNA expression were increased after the treatment.[195] The mechanism of action is suggested to be mediated by Bregs and antigen-specific Tregs.[196]

Antibody-based therapy

Apart from a peptide vaccine, several lines of evidence suggest that passive immunization, the administration with antibodies, against these epitopes could be effective too. Experimental results further support the idea of an antibody-based therapy against MDA-P45. By treating hypercholesterolemic mice with MDA-P45-IgG, a reduction of atherosclerosis with 50% could be achieved and similar observations have also been made in other mouse models.[197-199] Moreover, reduced levels of antibodies targeting MDA-modified p45 could be associated to increased CVD risk in clinical studies. [161,200] These finding gave rise to the idea that treatment with an antibody targeting this specific epitope represents a possible future therapy for treatment of CVD.

To study this further, carotid plaques from symptomatic patients were analysed and the results showed that the MDA-modified P45 epitope were present at a high degree in human atherosclerosis lesions.[201] The next step was to do an interventional clinical study. Thus, the GLACIER (Goal of oxidized LDL and activated macrophage inhibition by exposure to a recombinant antibody) trial was initiated, with the intention to test whether treatment with the antibody has protective effects. The GLACIER randomized-intervention trial consisted of 147 patients with stable coronary artery disease from 20 centres in the Unites States and Canada. However, the results indicated that the antibody did not reduce plaque inflammation and circulating inflammatory markers in these patients.[202] The lack of effect in this study might possibly be explained that the patients included might have been in a too stable state of disease to identify a response to treatment.

Treating atherosclerosis with anti-inflammatory drugs

As mentioned earlier, the overlapping pathogenesis of several autoimmune disorders sometimes appear to overlap. This provides an option to explore medications targeting several diseases. A successful example of this is the use of canakinumab, an anti-inflammatory immunomodulatory drug targeting the proinflammatory cytokine IL-1β in the treatment of RA. The CANTOS trial (Canakinumab Anti-Inflammatory Thrombosis Outcomes Study), a randomized

clinical trial assessing lowering of inflammation in patients with prevalent CVD, recently provided new evidence demonstrating the drug to be effective in lowering cardiovascular risk by reducing inflammation in cardiovascular events.[203]

Researchers with the interest to reduce inflammation in cardiovascular diseases have taken inspiration from studies proving an anti-inflammatory effect in autoimmune diseases such as RA. Methotrexate is highly efficient in the treatment of RA and is widely used in the treatment of these patients. Additionally, the drug seems to have protective effects in cardiovascular comorbidity and reduces the overall CVD burden in these patients according to one study. Following the CANTOS trial, the CIRT (Cardiovascular Inflammation Reduction Trial) study applied the same approach and sought to determine if a low-dose methotrexate might provide reduced inflammation in patients with stable atherosclerosis and T2D. Therefore, it was surprising that methotrexate did not result in a lower grade of inflammation in the CIRT trial individuals.[204,205]

Based on the increased incidence of of congestive heart failure (CHF) in RA patients treated with anti-TNF therapies, the question was raised regarding whether these drugs can modulate inflammation in CVD. Additionally, it was demonstrated that patients with CHF had increased levels of soluble TNF compared to healthy subjects. This was further associated with CHF event, and initiated clinical trials with the TNF inhibitor Infliximab and Etanercept. Disappointingly, those studies demonstrated no beneficial effects and unexpectedly there was a significantly increased mortality rate in patients receiving high dose of Infliximab, having side effects such as bradycardia and increased blood pressure. Of importance, other studies indicate that low levels of TNF are needed in the tissue repair processes after a heart failure.[206,207] These trials prove the importance of targeting the right pathway in atherosclerosis development and further highlight the diversity of inflammation presented in different diseases.

Clinical cohorts and experimental models

Clinical cohorts

Malmö Diet and Cancer Study

The Malmö Diet and Cancer Study[208] is a prospective population-based cohort consisting of approximately 30000 individuals from the Swedish city Malmö. The study was initiated in the 1990's with hopes to study environmental factors in relation to prediction of diseases such as CVD, diabetes and cancer. Subjects born between 1926 to 1945 and living in Malmö were eligible for inclusion in the study between October 1991 until February 1994. Additionally, every other participant that was included was invited to take part in the cardiovascular sub-study (Figure 5). This is the cohort used in **Paper 1**.

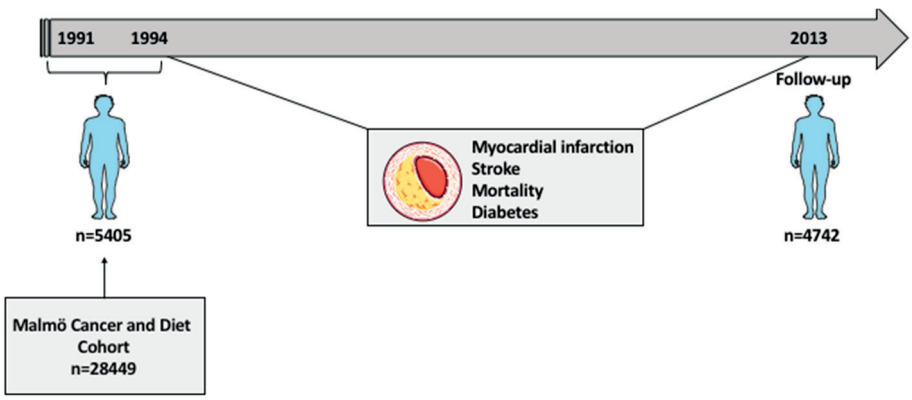

Figure 5. Study design of CVD subgroup from Malmö Cancer and Diet cohort All 45-73 year old men and women living in Malmö, Sweden were invited to participate in the study. A subgroup of 5405 individuals were enrolled in the cardiovascular subgroup.

The final number of participants for the subgroup was 5405 individuals, from whom fasting plasma samples and peripheral blood mononuclear cells (PBMC) were isolated and stored. 545 participants were excluded due to incomplete clinical data

and 118 individuals were excluded due to incomplete baseline biomarker measures. The remaining participants (n=4742) were followed from baseline examination until first event of a CVD event, emigration or death until December 31st 2013. A CVD event was defined as fatal or non-fatal myocardial infarction or ischemic stroke.

Karolinska Institute systemic lupus erythematosus cohort

The SLE cohort from Karolinska Institute, is an observational case-control study and the most extensive SLE cohort in Sweden. The cohort consists of 470 SLE cases and 322 controls and is also the cohort we base the studies in **Paper 2** on. All patients who received care for SLE at the Department of Rheumatology, Karolinska University Hospital in Solna, during January 2004 until October 2013 were asked to participate. The criteria to participate as a case were to fulfil four or more of the 1982 revised American College och Rheumatology (ACR) classification criteria for SLE[209] and a minimum age of 18 years. Criteria for the controls included maximum one year of age-difference, same gender and same region of living. A diagnosis of SLE was exclusion criterion.

Lund systemic lupus erythematosus cohort

To study the link between autoimmune inflammation and CVD, we used this SLE cohort to study the immune system and its responses against CVD-related antigens in **Paper 3**. The SLE cohort is an observational case-control study based on patients with prevalent SLE (n=53) with age and sex matched controls (n=32). Even though case-control studies comprise a lower number of participants, compared to a larger dynamic cohort, they provide a better view of the disease studies that are often rare or have a longer latency period. Additionally, it is cheaper and no follow-up time t to take into account which makes the sampling more convenient compared to a need for follow-up.

During 2014 to 2015, patients with prevalent SLE and age and sex matched controls (n=32) were enrolled at the Department of Rheumatology, Skåne University Hospital in Lund, Sweden. Clinical measurements of plasma markers and cytokines were obtained, together with a full assessment of the rheumatological features in SLE disease Activity Index (SLEDAI) 2000, Systemic Lupus International Collaborating Clinics (SLICC) and American College of Rheumatology Damage Index (ACR-DI). In addition, the patients completed questionnaires about lifestyle and general health. PBMC's from these individuals were isolated and stored for the investigation.

Experimental models

ApoE$^{-/-}$

One of the most commonly used experimental models of atherosclerosis is the ApoE$^{-/-}$ mouse. ApoE is present in all apolipoproteins and binds to the LDL-receptor. The ApoE$^{-/-}$ mouse model has a C57bl/7 background (B6), a common inbred laboratory mouse strain. Mice deficient in the ApoE gene have reduced ability to clear LDL-containing particles present from the circulation. Similar to hyperlipidaemia in humans, this causes chronic atherosclerotic and leads to plaque development when subjected to a high fat diet (HFD) that equals the Western type diet (21% fat, 0.15% cholesterol). This is a major modifiable risk factor for the disease development of atherosclerosis in humans. HFD introduction develops pronounced atherosclerotic lesions in the aorta of the mice, including the aortic root, arch and descending aorta. Additionally, ApoE$^{-/-}$ mice develop plaques in the subvalvular region of the heart that can easily be assessed by immunohistochemistry. Thus, the use of ApoE$^{-/-}$ mice isa good model to study the underlying mechanisms of atherosclerosis development. Most notably, the immune system is intact and can thus provide opportunities to investigate immunomodulation.

In the field of experimental atherosclerosis, it is well-known that female mice develop more atherosclerosis than male mice, which is also why it is considered to use females if possible.[210] This mouse model was used in **Paper 4**.

B6.lpr.ApoE$^{-/-}$

The B6.lpr.ApoE$^{-/-}$ mouse model was generated with the objective to have a model with excessive autoimmune inflammation in response to hyperlidipemia. In our B6.lpr.ApoE$^{-/}$ model, the SLE phenotype is based on the MLR/MpJ-Fas^{lpr} lymphoproliferation model which has a mutation in the gene encoding the Fas receptor. The MRL/MpJ strain itself is a model prone to autoimmunity regardless of the lpr mutation.[211]

When MLR/MpJ-Fas^{lpr} is crossed with the ApoE$^{-/-}$ on B6 background, the *lpr* mutation is inherited to the offspring but the MLR/MpJ strain is lost in favor of B6. This contributes to a B6 strain with lymphoproliferation and gives rise to an impaired clearance of antigens, dysfunctional apoptosis and loss of tolerance. This results in a mouse model with increased autoimmunity triggered by excessive lipids in the circulation. However, the SLE phenotype gets somewhat milder compared to

MLR/MpJ-Fas^{lpr} r since MRL is lost and the mice do not develop fatal kidney damage. This is an advantage in our experimental setting, where we mainly aimed to acquire autoimmune features in response to HFD in the cardiovascular system.

The B6.lpr.ApoE$^{-/-}$ model is similar to the B6.gld.ApoE$^{-/-}$ model used by Aprahamian et al., who have performed similar studies on autoimmunity manifested in the cardiovascular organs. The two models give rise to highly similar SLE phenotypes, with the only difference that *gld* stands for a mutation of the Fas Ligand and not Fas receptor.[212]

Results and discussion of present investigation

Paper 1

Elevated markers of death receptor-activated apoptosis are associated with increased risk for development of diabetes and cardiovascular disease

Atherosclerosis is affected by several environmental factors including metabolic stress, chronic vascular injury and apoptosis.[213] In **Paper 1**, we hypothesized that circulating death receptors in the plasma are markers of receptor-activated apoptosis and could predict risk for development of diabetes and cardiovascular events (Figure 6). This possibility was investigated *in vitro* and in clinical samples. The circulating death receptors TNFR-1, Fas and TRAILR-2 were detected in plasma from 4742 individuals in the Malmö Diet and Cancer Study in relation to future diabetes and cardiovascular events.

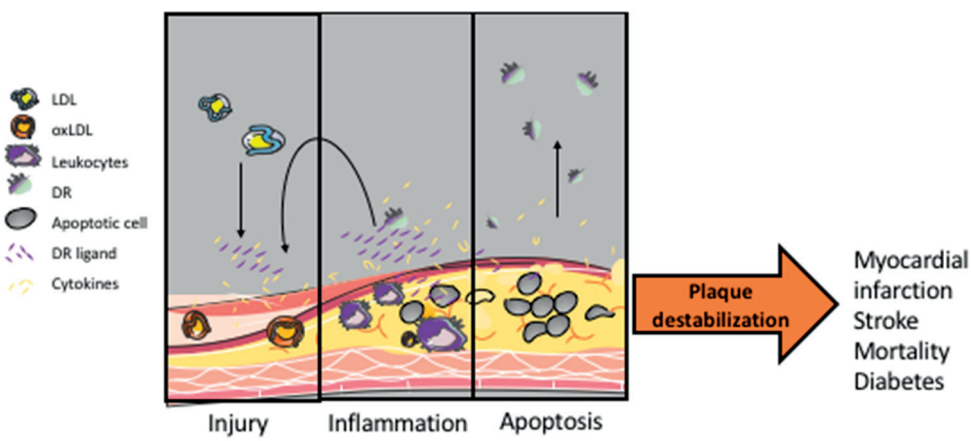

Figure 6. Postulated hypothesis for Paper 1. Circulating death receptors in the plasma are markers of receptor-activated apoptosis and could predict risk for development of diabetes and cardiovascular events.

Importantly and highly relevant in this context, is the assembling event between extracellular death receptor domain and the soluble ligand. When the binding occurs a shedding mechanism of the extracellular receptor domain occurs simultaneously.[74] Thus, detection of soluble TNFR-1, Fas, and TRAILR-2 in the plasma gives a quantitative measure of death receptor activated-apoptosis.

In **Paper 1**, we investigated if induced death receptor activated-apoptosis through stimulation with known ligands, IL-1β, Fas Ligand and TNF, could mediate shedding of the extracellular domain from PBMC's and β-cells *in vitro* as the literature proposes. In both cell types, a dose-dependent significant increase of the death receptors TNFR-1, Fas and TRAILR-2 could be detected after stimulation with soluble Fas Ligand, but not with Fas and TNF.

Table 2. Correlations between markers of death receptor-activated apoptosis and cardiovascular risk factors at baseline in non-diabetic subjects.

	TNFR1	p	FAS	p	TRAILR2	p
Age	0.22	<0.001	0.20	<0.001	0.23	<0.001
Glucose	0.13	<0.001	0.14	<0.001	0.12	<0.001
Insulin	0.19	<0.001	0.19	<0.001	0.17	<0.001
BMI	0.20	<0.001	0.17	<0.001	0.13	<0.001
Triglycerides	0.19	<0.001	0.19	<0.001	0.20	<0.001
HDL	- 0.21	<0.001	−0.17	<0.001	−0.18	<0.001
LDL	0.06	0.001	0.10	<0.001	0.08	<0.001
Systolic BP	0.15	<0.001	0.13	<0.001	0.13	<0.001
hsCRP	0.23	<0.001	0.09	<0.001	0.22	<0.001

Correlation coefficients were calculated using Spearman Rank test. BP;blood pressure.

This encouraged us to look closer into the plasma levels of soluble death receptors in a subset of the Malmö Diet and Cancer Cohort (N=4742), a prospective population-based cohort with a follow-up period of 19.3 years. After exclusion of prevalent diabetic subjects at baseline, a significant association were shown between the three death receptors of interest and metabolic/CVD risk factors (Table 2).

Interestingly, subjects with prevalent diabetes (n=363) had significantly higher levels of the three circulating death receptors of interest compared to non-diabetics (n=4379). Similarly, current smokers (n=951) also had significantly higher levels of all death receptors compared to non-smokers n=3791. These results suggest that the increased burden of cellular and metabolic stress also increases death receptor-activated apoptosis.

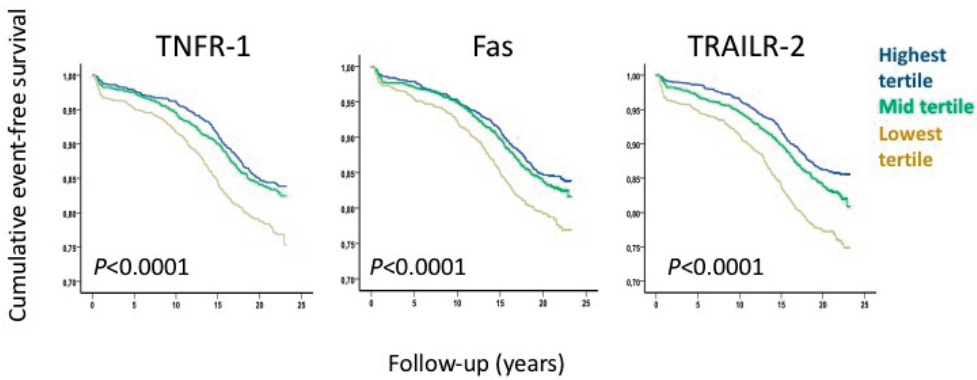

Figure 7. Myocardial infarction in non-diabetic patients have an association with death receptor concentration
Kaplan-Meier plots presenting the associations between myocardial infarction and tertiles of the death receptors TNFR1, Fas and TRAILR2 plasma levels for non-diabetic patients. P<0.0001, p-values were calculated using Spearmans log rank test.

In Cox Regression models, the highest tertile of each of the death receptors at baseline in the non-diabetics could not only predict future events in terms of myocardial infarction (Figure 7), but also stroke and all-cause mortality (data not shown). When adjusting for risk factors, the association remained statistically significant for TNFR-1 (MI and death) and TRAILR-2 (MI, stroke and death), but not for Fas death receptor. It is important to note that the levels of death receptors were not genetically influenced as a genome-wide association study revealed none or very minor associations remained after adjustments for the same risk factors used in the analysis above mentioned.

Several limitations of the study need to be taken into account. If our hypothesis that death receptors reflects the death receptor-activated apoptosis is correct, the subjects in the study would express additionally higher levels at the actual time of the event. Unfortunately, we cannot confirm or exclude if possible changes in the plasma levels of these apoptosis markers has influenced the results.

Conclusion

These results show that elevated death receptors via cellular stress is associated with future CVD events and thus probably atherosclerosis progression.

Although we did not prove a causal relationship between death receptors and event, the identified associations strongly suggest an involvement of death receptor associated-apoptosis in atherosclerotic plaque injury. herein, our results provide clinical support for the response to injury hypothesis and thus, we believe that measurement of death receptors might reflect the current status of cellular stress and might be a good way to monitor the patient's response to treatment.

Adjusting for the right variables without distorting the biology

An important step in epidemiological studies is to adjust for relevant risk factors in order to avoid confounding effects of the variables studied. In **Paper 1**, we adjusted for age, glucose, body-mass index(BMI), HDL, LDL, gender, triglycerides, high-sensitivity C-reactive protein (hsCRP), systolic blood pressure (sBP) and smoking. If adjusting for too many covariates the results might be biased. One example of over-adjustment is to correct for birth weight in a regression analysis with the association between maternal smoking and neonatal mortality. Maternal smoking is considered to mediate a decreased birth weight. Conversely, adjustment for birth weight would represent overadjustment.[214]

On the other hand, if no adjustment for confounding factors is performed the biology might be skewed in the other direction. This can be exemplified in a study exploring the possibility that consumption of chocolate leads to better cognitive function which in turn, reflects the higher predominance to winning a Nobel Prize. Hence, one study published in the New England Journal of Medicine in 2012 hypothesizes that a country's level of chocolate intake correlates with its total number of Nobel laureates per capita. In fact, a significant correlation between chocolate and Nobel prize winners in Switzerland was observed ($r=0.791$, $p<0.0001$) which indeed can be interpreted as that chocolate consumption increases the chances of winning a Nobel Prize.[215] In this study, the results are influenced by obvious systematic errors with confounders such as wealth. Additionally, the Swiss author disclosed a daily chocolate consumption, which itself reveals a suspected confirmation bias (a tendency to search for results to confirm pre-existing beliefs). Of course, random errors such as genetic drift or measurement fluctuations always occur, but these can't be controlled in a statistical model such as systematic errors.

In the study population described in in **Paper 1**, inflammatory mediators such as LDL are entrapped in the plaque and initiates immune responses. During atherosclerosis, the inflammation cannot be fully resolved, which is causative for the apoptosis that immune cells undergoes as well as for the formation of pathological necrotic cores. This apoptosis event mediates death receptor release. Thus, LDL is not only a covariate affecting the CVD but also the apoptosis, making. LDL a confounding variable that needs to be adjusted for when exploring independent associations.

Death receptors reflects tissue injury

In **Paper 1**, we demonstrate *in vitro* that death receptor-activated apoptosis through mediated by soluble Fas ligand significantly increases the release of soluble death receptors TNFR-1, Fas and TRAILR-2. We also show a link between death receptor-activated apoptosis and future risk for development of diabetes and CVD.

This is emphasized by the observation that current smokers had higher levels of soluble death receptors in their plasma, compared to non-smokers. Smoking causes cellular stress systemically, similar to metabolic factors, and reflects cell death through death receptor-activated apoptosis based on the increased circulating death receptors. Therefore, it is likely that death receptors may be markers of the ongoing tissue injury.

The cellular stress fits well into the Response-to-injury hypothesis. Ross and Glomset postulated that atherosclerosis is developed because of a response to the lipids in the arterial wall causing injury. Inevitably, that will mediate cellular stress followed by apoptosis of the stressed cells. In support of the hypothesis that death receptor mediated apoptosis causes cellular stress similar to the injury caused by lipids, the apoptosis markers Fas-associated death domain (FADD), caspase-3 and caspase-8 can be significantly associated with the incidence of coronary events in the same cohort.[216]

The association between death receptor activated-apoptosis and cardiovascular disease events

The associations between the death receptors TNFR-1, Fas and TRAILR-2 were significantly associated with CVD events when excluding prevalent diabetes. The reason for excluding individuals with prevalent diabetes is the already heightened cellular stress in these patients, which is present despite a CVD event.

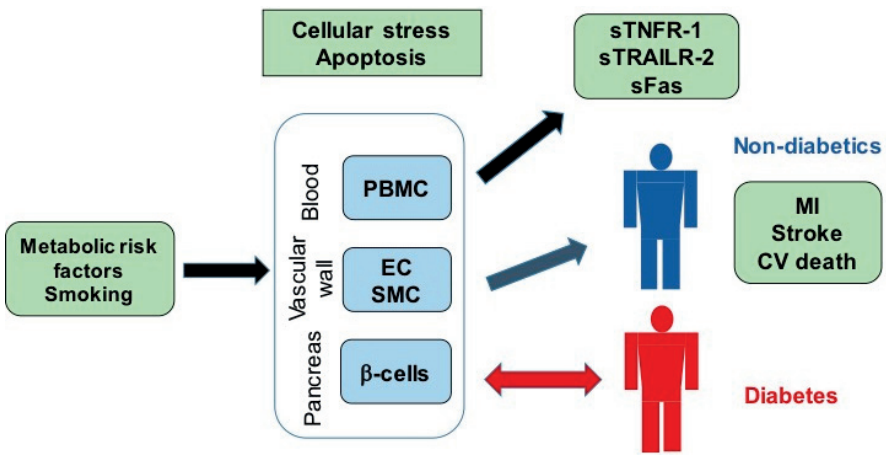

Figure 8. Association between metabolic stress, relese of soluble death receptors and development of diabetes and cardiovascular disease. Smoking and metabolic risk factors such as hyperglycemia, hypertriglycemia and low HDL cause cellular stress leading to increased cell death through death receptor-activated apoptosis in exposed tissues. The activation of death receptor-activated apoptosis is associated with release of soluble (s) TNFR-1, sFas and sTRAILR-2 into the circulation suggesting that the plasma level os these receptors act as a marker of the tissue injury caused by the risk factors. When pancreatic β-cekks are affecgted the risk for development of diabetes increases while injury to vascular cells promotes atherosclerosis and increases the risk for cardiovascular events.

Our study does not provide evidence of a causal relationship between elevated death receptors and future CVD event, but is supported by the associations that metabolic risk factors is a contributing factor to CVD. The role of death receptors in cellular function in the immune system is still controversial as activation of death receptors also have been described to result in inflammation and cell proliferation.[217] This, however, is not in line with the shedding-theory[74] that suggests an initial apoptotic process rather than activation of inflammation. The direction of events in a complex process such as atherosclerosis is hard to prove, and in spite of significant associations observed reverse causation cannot be ruled out.

Paper 2

Cardiovascular disease in systemic lupus erythematosus is associated with increased levels of biomarkers reflecting receptor-activated apoptosis

A key factor in both SLE and atherosclerosis is the impaired regulation of apoptosis.[218,219] In SLE, it is well-established that the clearance of apoptotic material is dysfunctional. [220] Similarly, apoptotic cells entrapped in an atherosclerotic plaque, cannot be cleared properly. The remaining debris of apoptotic material is highly immunogenic and mediates a pro-inflammatory milieu.[220,221] In **Paper 2**, our objective was to investigate if biomarkers of four aspects of autoimmunity could be associated to CVD in subjects with SLE. Therefore, we hypothesized that markers of apoptosis, inflammation, tissue degradation and repair reflected CVD in SLE individuals (Figure 9).

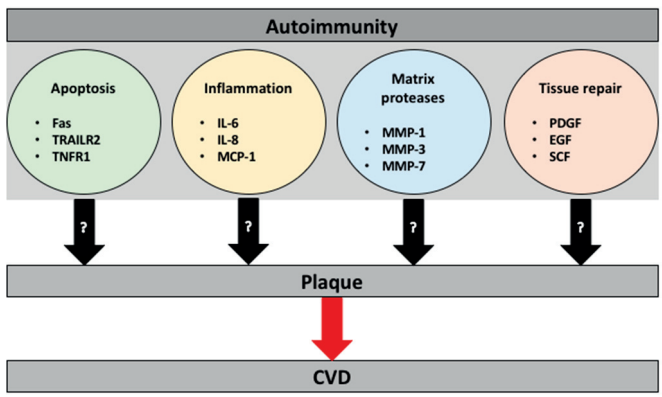

Figure 9. Overview of biomarkers of interest and possible link in CVD. Autoimmunity is a driving mechanism in plaque progression and CVD. The four aspects of apoptosis, inflammation, matrix proteases and tissue repair was studied in Paper 2.

In **Paper 2**, biomarker levels in SLE subject with prevalent CVD (n=69) were compares to those without CVD (n=401). Additionally, the biomarker levels in SLE subjects with atherosclerotic plaques (n=60) were compared to those without plaque (n=236). CVD was defined as history of objectively verified coronary artery disease, ischemic cerebrovascular disease or peripheral artery disease that was determined by reviewing medical files. The plaque burden was determined as carotid ultrasound detection of the carotid intima-media thickness. Comparisons between CVD versus non-CVD and plaque versus no plaque respectively, showed highest significances of apoptosis markers (Table 3). Further, only TRAILR-2 remained significant after adjusting for Framingham risk factors, similarly to our results in **Paper 1**.

Table 3. Biomarker levels in SLE patients with and without CVD

	No CVD (n = 401)	CVD (n = 69)	*p*	No plaque (n = 236)	Plaque (n = 60)	*p*
Apoptosis						
Fas	177 (141–229)	209 (162–272)	.004	174 (134–222)	220 (170–270)	<.001
TRAIL receptor-2	2.41 (1.85–3.39)	3.32 (2.19–4.48)	<.001	2.21 (1.64–2.86)	2.96 (1.82–4.14)	<.001
TNF receptor-1	5557 (4513–7564)	6562 (5480–9090)	.001	5293 (4421–6985)	6631 (5349–9027)	<.001
Inflammation						
IL-6	37.8 (20.8–74.0)	52.3 (30.0–84.7)	ns	35.9 (20.4–59.5)	45.6 (28.3–81.99)	ns
IL-8	37.3 (27.4–57.7)	47.5 (33.4–71.5)	ns	37.4 (30.0–56.1)	57.1 (35.2–81.8)	.001
MCP-1	7.52 (5.47–10.70)	9.00 (6.43–11.91)	ns	7.52 (5.52–11.16)	9.29 (6.48–12.51)	ns
Matrix proteases						
MMP-1	6.92 (3.99–12.42)	7.46 (4.61–18.26)	.01	6.73 (4.11–12.53)	11.08 (5.34–16.17)	.003
MMP-3	2.08 (1.23–3.80)	2.25 (1.41–3.69)	ns	1.72 (1.17–3.61)	2.26 (1.41–3.82)	ns
MMP-7	218 (165–312)	286 (218–422)	.001	220 (169–300)	287 (221–430)	<.001
Tissue repair						
PDGF	132 (78–222)	121 (59–250)	ns	125 (71–215)	156 (89–283)	ns
EGF	66.7 (38.0–112.2)	53.4 (29.6–122.4)	ns	61.4 (31.8–99.6)	62.7 (33.8–117.4)	ns
SCF	119 (92–152)	125 (101–157)	ns	122 (93–153)	117 (99–143)	ns

Distributions are given as median (interquartile range) or percentages. CVD data missing for 14 SLE patients. ns; not significant.

Conclusion

Our findings in **Paper 2** demonstrate that soluble death receptors systemically increase in SLE patients with cardiovascular complications. The findings also suggest that an intensified signalling through death receptors may contributes to the cardiovascular complications, including elevated apoptosis in SLE subjects. Collectively, in **Paper 2** we show an association between soluble death receptors and CVD in SLE patients. Similar to **Paper 1**, we suggest that plasma levels of soluble death receptors reflect apoptosis linked to atherosclerosis development.

The effect of lipid-lowering treatment in SLE patients

Lipid-lowering drugs might affect the levels of inflammation markers.[222] This motivated us to look into if treatment with lipid lowering drugs had an effect on inflammation markers in prevalent CVD and plaque burden. Unexpectedly, we discovered that individuals treated with lipid lowering medications (n=54) also had higher levels of IL-6, IL-8 and monocyte chemoattractant protein 1 (MCP-1, p=0.01, p=0.01 and p=0.001, respectively). As compared to research in non-SLE

individuals, who show a beneficial effect of lipid lowering drugs[223], these results show the opposite.

To date, there are no published studies focusing on possible adverse effects causing inflammation in SLE subjects. However, there have been a handful studies on statin-induced immune-mediated myopathy, with autoimmune features. These subjects display classical symptoms of cardiomyopathy together with an autoimmune profile with circulating autoantibodies.[224] The use of statins is common in SLE patients as preventive therapy due to their increased risk of CVD.[225] Because of the frequent usage, the characterization of possible adverse effects is still warranted.

Limitations of *in vitro* experiments

In **Paper 2**, we found associations between apoptosis markers and CVD in subjects with SLE. To further address how apoptosis markers are associated with CVD in SLE patients, we stimulated PBMCs from SLE and control individuals with soluble Fas Ligand, which we in **Paper 1** showed could induce death receptor-apoptosis. However, the effects on death receptor release or apoptosis in **Paper 2** were not as clear as the clinical studies.

One common problem with the use of PBMC's is the variability of cryopreserved samples. One study explored the recovery of frozen PBMC's and reported differences in the viability for every 10 fold decrease.[226] That might be one contributing factor to a less pronounced response from the SLE PBMC's compared to the controls.

It is not unusual that SLE patients are treated with immunosuppressive, anti-inflammatory or steroid-based therapies. All these medications result in a compromised immune system. Based on our study summarized in **Paper 2**, we did observe a lower yield of SLE PBMC's compared to controls. This was resolved by adjusting the cell number to the lowest number of cells obtained, but this does not reflect the recovery. Most likely, the control PBMC's experienced a better thawing procedure with higher viability compared to the SLE PBMC's that might have had an effect of the apoptosis activation in **Paper 2.**

Paper 3

Two immunomodulatory approaches to raise atheroprotection against the Apolipoprotein B 100 peptide p45 in a hypercholesterolemic murine model of systemic lupus erythematosus

Because CVD is the main cause of death in SLE, where traditional CVD therapies are unsuccessful, there is a large need for novel treatment strategies.[191] An immunization strategy to selectively target the antigen-specific protection without any irrelevant side effects would be a major contribution to future precision medicine. In **Paper 3**, we hypothesize that atherogenesis in SLE in part is driven by autoimmune responses against atherogenic antigens such as oxLDL.

Based on the systemic lupus erythematosus cohort from Lund University, we detected an increased inflammatory profile, where baseline plasma levels of IL-6 correlated to oxLDL ($r=0.32$, $p<0.05$) in SLE individuals but not in controls. To analyse if oxLDL induced proinflammatory responses in SLE individuals, we next incubated PBMCs with oxLDL (10µg/ml) *in vitro* for 24h. We could show that oxLDL activates immune cells and induced inducible T-cell costimulator (ICOS) positive APC population (defined as $ICOS^+$ of $CD3^-ZombieAqua^+$)

These results lead us to test two different immunomodulatory approaches in a hypercholesterolemic SLE mouse model targeting a peptide sequence presentin ApoB100. The first approach to modulate the immune responses against oxLDL was to test if P45 coupled to CTB could induce tolerance against P45 using oral immunization (Figure 10 A), as was shown in other studies using P210 as an antigen.[185,195] In **Paper 3**, our findings suggest that the antigen memory is induced in the mesenteric draining lymph nodes after P45-CTB immunizations (Figure 10 B). Most importantly, the results showed a reduced plaque progression in aorta and subvalvular lesions as well as less macrophages in the subvalvular plaques (Figure 10 C). However, no specific-antigen response could be detected in plasma, even though there was activation of B cellsin response to the vaccine (Figure 10 D). In our second approach of **Paper 3**, an IgG antibody targeting MDA-modified P45 (MDA.P45-IgG) was administered after high fat diet (figure 11 A). Interestingly, the subvalvular plaque area, CD68 percentage and the aortic arch lipid area were reduced compared to mice treated with a control IgG (Figure 11 B-C).

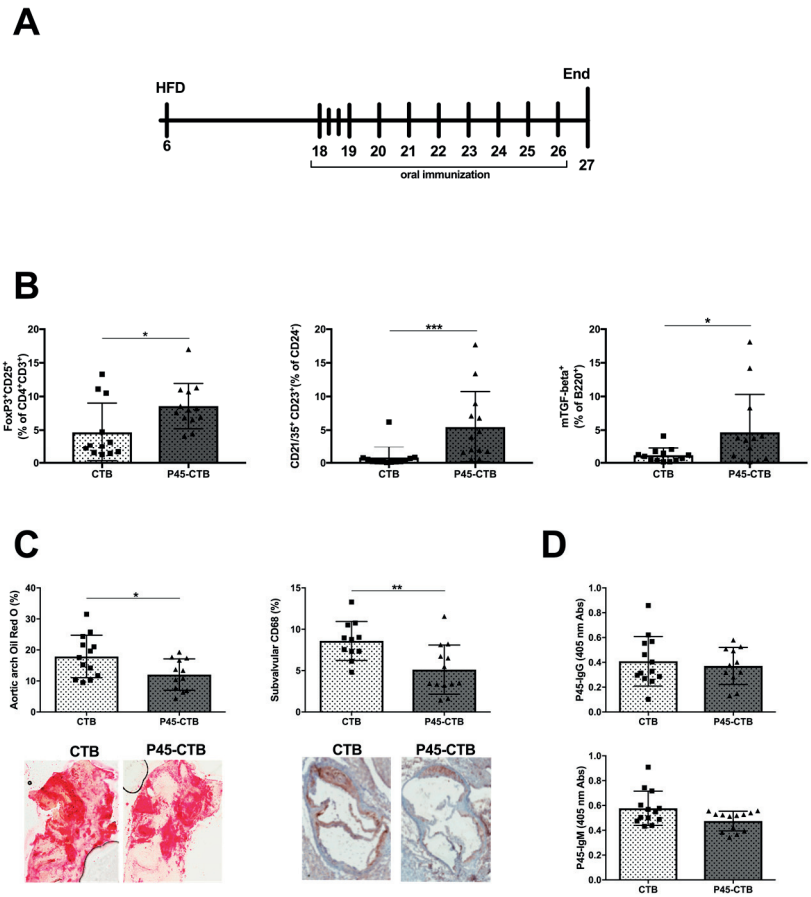

Figure 10. Oral immunization with P45-CTB (A) Experimental outline of the study. (B) Immune cell subsets from draining mesenteric lymph node at the time of sacrifice, (C) aortic arch atherosclerotic lesion amount and subvalvular CD68 macrophage amount and (D) plasma P45-antibody responses.

These results brought up the question whether the antibody could exert similar mechanisms in SLE individuals that have an impaired antibody production.[227] To further investigate this, we incubated PBMC's with oxLDL or oxLDL/ MDA.P45-IgG. Interestingly, the overall uptake of oxLDL was induced with the antibody present (defined as DILoxLDL^{+} mean fluorescence intensity, MFI). Moreover, the results suggest that the CD14^{+} cells were responsible for the uptake and abolishment of oxLDL.

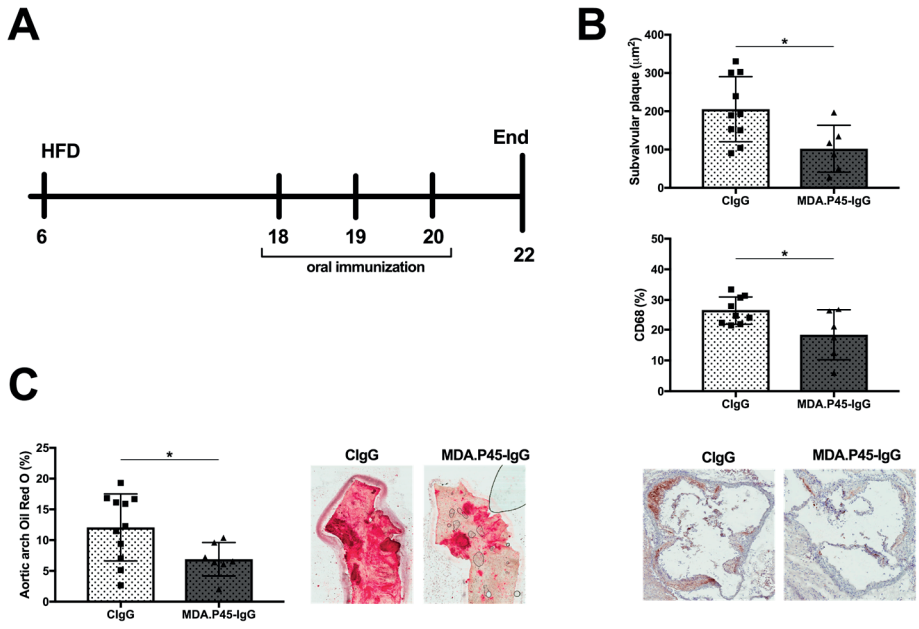

Figure 11.Passive immunization with MDA.P45-IgG (A) Experimental outline of the study. (B) and subvalvular plaque area and CD68 macrophage amount. (C) aortic arch atherosclerotic lesion amount.

Conclusion

To summarize **Paper 3**, we demonstrate how oxLDL affects PBMC immune responses in healthy controls and SLE cases. Experimentally, we show two strategies to modulate the immune responses in hypercholesterolemic SLE mice. Firstly, how P45-CTB oral immunization induces a regulatory T cell response in these mice. Secondly, we conclude that passive immunization with MDA.P45-IgG successfully reduces the lesion size in the same mouse model. Finally, we show how MDA.P45-IgG affects a cell population of importance in the uptake and clearance of oxLDL in human control and SLE PBMCs. Collectively, these findings suggest that targeting immune responses against oxLDL could represent possible approach for prevention of CVD in SLE.

Technical limitations in the study design

There are limitations in the design of this study to consider. One limitation is that we did not combine our treatment with another anti-inflammatory drug such as corticosteroids or immunomodulators which SLE patients are most often treated with long term (previously mentioned as a central limitation of the *in vitro* studies in **Paper 2**). Within our patient cohort, almost all of the patients are treated with at least one drug targeting inflammation. With this, the beneficial effects of the

immunomodulatory treatments observed might have been overridden in the *in vitro* experiments.

Another limitation in this study is the possibility of cross-reactivity in the assay used to detect P45-antibodies. Other autoantibodies in plasma from the mice might have caused false-positive results. The absorbance signal of P45-IgG/IgM would be diminished. This phenomenon is frequently observed in other serological clinical SLE tests due to the nature of the higher affinity of autoantibodies in SLE.[228] Our in-house assay is not optimized for those conditions, and the fact that the background was relatively high contributes to these concerns.

Proposed mechanism of action for MDA.P45-IgG

Previous studies with MDA.P45-IgG have provided evidence of anti-inflammatory effects on macrophages in the atherosclerotic plaque.[197] In addition to a decreased amount of CD68 or MOMA-staining in the plaque, functional studies have also show that the antibody engages with FcγR. This interaction exerts anti-inflammatory mechanisms and a decreased pro-inflammatory cytokine secretion that we also were able to confirm in Paper 3.[229] Moreover, it is likely that the effects are mediated through FcγRIIb, the only FcR with inhibitory effects.[202,230] This receptor is upregulated on monocytes by pro-inflammatory cytokines.[231,232] With this knowledge, in combination with present investigations from Paper 3, our proposed mechanism of action for MDA.P45-IgG is an uptake in $CD14^+$ cells that in turn become of an anti-inflammatory nature (Figure 12). Possibly, the oxLDL-antibody complex promotes an increased FcγRII-mediated uptake which results in lower uptake by scavenger-receptors.

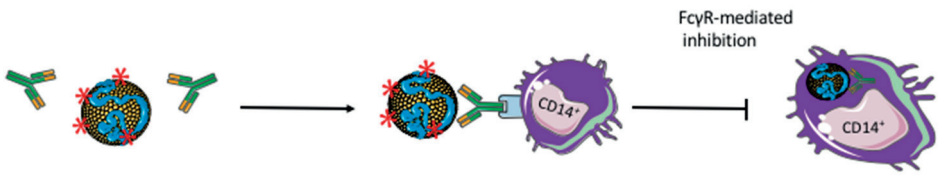

Figure 12. Proposed mechanism of action for MDA-P45-IgG The antibody targets MDA-modificed P45 present in oxLDL particles. The complex is taken up by FcγRII receptors on $CD14^+$ macrophages. Further, the engagement with FcγRII exerts inhibitory effects on the macrophage that takes up the oxLDL-antibody complex for abolishment.

Further, it is widely known that unstable atherosclerotic plaques contain more monocytes/macrophages compared to stable plaques.[233] If the proposed mechanism is true, it would also explain the results from the GLACIER trial, in which the patients were in a stable phase of CVD and possibly did not have sufficient plaque inflammation to modulate.[202]

Advantages and disadvantages with a vaccine in an autoimmune setting

There is more than one immune pathway to consider when aiming to induce atheroprotection. Tregs have a well-documented function of exerting protective functions against atherosclerosis. For example, prospective clinical studies report that the presence of low levels of Tregs are associated with increased risk for development of CVD.[234,235 236]

In the first immunomodulatory approach in **Paper 3**, oral administration with P45-CTB generated regulatory T cells and protective mTGFβ^{+} B cells when inducing tolerance with CTB.[193,237] One advantage with this vaccine strategy is the route of administration. With an oral vaccine, the vaccine can be administered safely several times without the need for clinical visits. The fact that peptides are cheaper to generate compared to antibodies is another important aspect to take in mind. However, this vaccine approach also has some limitations that needs to be considered. The absence of a specific antibody response in our setting might be a result of the SLE phenotype, since the B cells in the *lpr*-model lacks a fundamental regulatory function.[238] If the absence of an antibody response is caused by the SLE disease, it is a disadvantage. It is also not possible to know if the same effects would be present in a human setting. A regulatory response alone might benefit the patient, but may not be sufficient for atherosclerosis without a specific antibody response. Therefore, no conclusions can be drawn about a possible antibody protection without more research.

In the second immunomodulatory approach used in **Paper 3**, we investigated possible protective actions of MDA.P45-IgG, a monoclonal antibody directed against the MDA-modified peptide p45 in ApoB100. One benefit with an antibody therapy is the selective specificity for the antigen, without engaging the adaptive immunity which may be more suitable for individuals that have a dysfunctional adaptive immunity. If an antigen-specific response is the missing factor into protection against oxLDL in SLE individuals, it may be restored with an antibody therapy. Even though there is a rationale why this antibody may have beneficial effect in SLE, an antibody therapy could seem counter-intuitive as a therapeutic option because the pathogenic nature of the autoantibodies present in these individuals. Nonetheless, the fact that SLE individuals have an impaired removal of apoptotic material as well as immune complexes through macrophage scavenger receptors contributing to autoimmunity,[239] makes as strong case for the consideration of an antibody-therapy to reduce CVD risk in SLE patients. Moreover, this specific antibody seems to be present at lower levels in SLE subjects which further supports a potential beneficial role. As a therapy.[177] To rule out detrimental effects caused by the antibody treatment, we chose to review possible inflammation in the kidneys caused by immune complexes form the circulation. The kidneys are

usually the first organ to be affected in SLE.[240] Though, the mice in the study did not express any adverse effects and had no organ damage detected in the kidneys.

From **Paper 3** we can conclude that both immunization strategies are safe in the mouse model used in our experiments and does not aggravate inflammation. The mechanism of action and the efficacy if both treatment strategies for SLE are still elusive and require further research. Both approaches have their individual positive and negative aspects important to take in mind. At this moment, we cannot conclude if one is more efficient in targeting inflammation in atherosclerosis development and meanwhile provokes less side-effects than the other. Thus, more research is needed to evaluate the effects in a human setting.

Paper 4

B cells treated with CTB-p210 acquire a regulatory phenotype *in vitro* and reduce atherosclerosis in apolipoprotein E deficient mice

Similar to p45 of ApoB100, the peptide 210 (P210) is an epitope present in LDL. This peptide has been shown to induce adaptive immune responses resulting in reduced plaque development.[241] In $ApoE^{-/-}$ mice, intranasal immunization with P210-CTB induced antigen-specific Tregs.[195] Similarly, subcutaneous administration of the native peptide reduced atherosclerotic lesions and increased the Treg population.[235] These findings support the possibility of generating a mucosal vaccine targeting atherosclerosis Therefore, we aimed to investigate if P210-CTB has protective effects against atherosclerosis and if these effects are mediated by Bregs in Paper 4.

In line with previous findings, we were able to induce membrane $TGF\beta^{+}$ ($mTGF\beta^{+}$) B cells and $CD25^{hi}FoxP3^{+}$ T cells after exposing $Apob/LDLR^{-/-}$ or $ApoE^{-/-}$ naïve B cells and T cells with P210-CTB in Paper 4 (Figure 13 A-B). Based on this, we could confirm the regulatory effects of P210-CTB exposure *in vitro* and further wanted to evaluate those effects *in vivo*. P210-CTB or ovalbumin-CTB (OVA-CTB) pulsed B cells from $ApoE^{-/-}$ on high fat diet, were adoptively transferred three times to $ApoE^{-/-}$ mice. Mice that received the modified B cells, revealed a significant decrease in the aortic plaque area (Figure 13 C) and an increased plasma level of anti-P210 IgG. However, no effects of pro- or anti-inflammatory features were detected in subvalvular plaques, splenocyte cell populations or carotid mRNA gene expression.

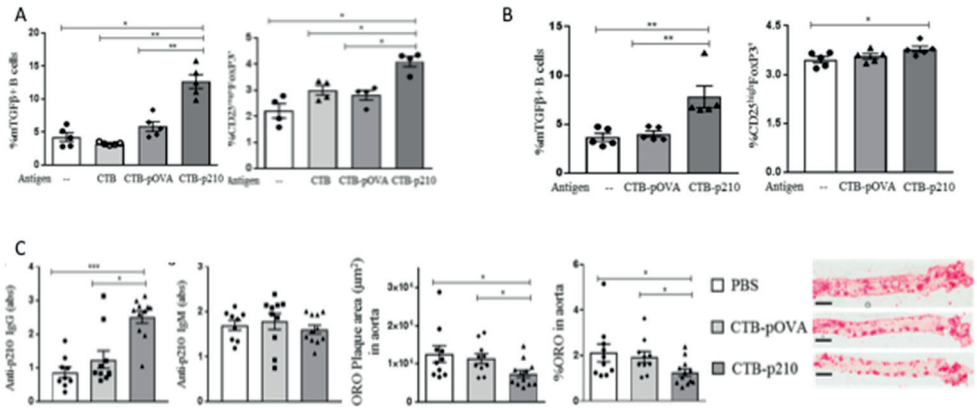

Figure 13. P210-CTB induced Bregs and Tregs *in vitro*. P210-CTB induced mTGFβ⁺ B cells and CD25^{hi}FoxP3⁺ T cells from naïve B and T cells isolated from (A) Apob/LDLR$^{-/-}$ on high fat diet or (B) ApoE$^{-/-}$ mice on high fat diet. (C) Plasma anti-P210 IgG and IgM and aorta oil Red O staining including representative pictures. Oil Red O data was log trsnformed before statistical analysis.

Conclusion

In **paper 4**, we conclude that B cells pulsed with P210-CTB *in vitro* acquire a regulatory phenotype, as seen by the increased Breg and Treg numbers. Further, adoptive transfer of these B cells induced an antigen-specific response and decreased atherosclerosis *in vivo*. The suppressive effects elicited by the B cells after P210-CTB exposure suggests that Bregs are involved in the induction of tolerance against P210 of ApoB100.

Immunogenicity of the peptide

Oral tolerance is defined as suppressive immune responses to antigens given by oral rout of administration. The mucosal barrier in the intestines work in concert with lymphoid cells to promote a defence against pathogens while at the same time preserving tolerance for food-related antigens. Induction of antigen-specific tolerance through oral immunization mediates a strong tolerogenicity due to the immediate encounter with APC's.[242] Though, an adjuvant is needed to oversee the mucosal surface to provide a site for antigen attachment to be available for the immune cells.[182] CTB, with its high membrane-binding capacity, is used as an adjuvant. CTB mediates binding to the ganglioside GM1 receptors on the gut cells where the antigen is exposed to the APCs located in the gut-associated lymphoid tissues (GALT).[192,242] This induces antigen-specific T cells that strongly suppresses antigen-activated effector T cells.[193] The suppressive effects are believed to act through enhanced IL-10 and TGF-β production.[193] In **Paper 4**, we could however only show a Treg response *in vitro*, but not *in vivo*.

One possible interpretation to the lack of *in vivo* Treg response in **Paper 4**, could be insufficient immunogenic properties of the peptides to GALT. As reviewed in Table 3, there are several other antigens that have been studied in the context of atheroprotection, where some of them have been able to successfully induce antigen-specific Tregs. Similar to our approach using a peptide-based formula to induce oral tolerance, another study showed an induction of oral tolerance with an HSP60-peptide. Indeed, a Treg response could be observed and the plaque area correlated with the Tregs numbers as well as a regulatory cytokine profile.[108] Interestingly, the heat-shock protein 60 (HSP60) peptide is highly conserved throughout different pathogenic species and resembles bacterial PAMP's. The HSP60 peptide may thus be confused with a pathogen resulting in activation of an innate autoimmune response. This confusion is known as molecular mimicry, and mediates a powerful immunogenic response. Molecular mimicry and resemblance with a PAMP might thus be crucial for a robust induction of a Treg response similar to an adjuvant.[243]

To induce Tregs, it is critical that the peptide is presented on a MHC II to be able to ligate with a TCR and elicit regulatory responses.[244] In **Paper 4**, we failed to induce Tregs *in vivo* and the lack of molecular mimicry with a pathogen could be one reason for this. Instead, the peptide might have been cleared out rather than presented to the T cells. [245] To overcome this, the delivery of the peptide should be overseen and directed to facilitate TCR encounter.

Summary and future perspectives

Can immune-modulatory treatment provide additional benefit to cardiovascular patients?

Atherosclerosis is a chronic inflammatory disease mediated by excessive lipid burden in the vessel. The studies presented in this thesis focuses on the immune responses of the disease, and possible new immune-based therapeutic approaches. One of the major problems today is that we detect the disease too late. Another problem is that the current therapies does not target all mechanisms of the disease. Today we treat the patients according to the symptoms they experience as a result of manifest disease. If a patient is identified according to the traditional risk factors, such as high level of lipids, blood pressure or overweight, atherosclerosis has already affected the patient internally. In the long run, identification of individuals at risk before the process has progressed too far would spare lives and pain. This can be resolved by identification of patients at risk. If the early immunological mechanisms of the disease can be targeted therapeutically, there are greater chances of survival (Figure 14).

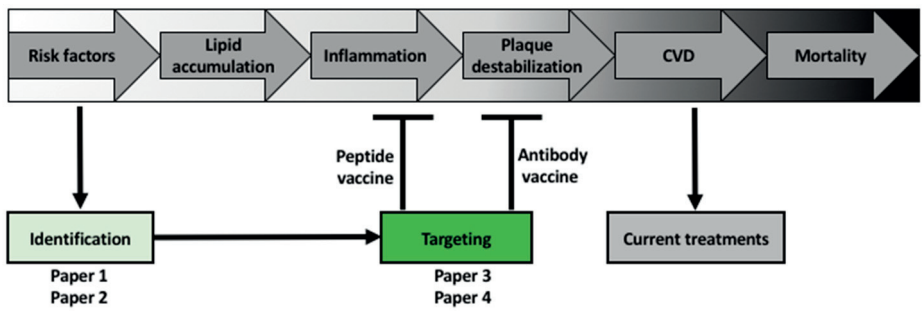

Figure 14. Identification and targeting of patients at risk before CVD events. The assessment of risk factors and biomarkers reflecting these can be used as a tool to target patients at risk. The use of immunomodulatory therapies targeting the underlying inflammation of atherosclerosis would benefit those patients greatly.

The first part of this thesis addresses novel biomarkers and their association to certain CVD events. In **Paper 1**, we show that circulating death receptors in the plasma can be used in the identification of receptor-activated apoptosis and that this is a predicting risk for development of diabetes and cardiovascular events. Similarly, in **Paper 2** we conclude that circulating death receptors in the plasma also can be associated to CVD in patients with SLE. **Paper 1 and 2** together provide evidence for an association between apoptotic cell death and risk for development of CVD and that this association is of particular importance in patients with autoimmune disorders such as SLE.

In **Paper 3 and 4**, the mechanisms of immune responses in atherosclerosis and possible immunomodulating therapies are investigated. Because our findings from Paper 1 and 2 implied that atherosclerosis might be influenced by autoimmune mechanisms, we chose to continue to study the responses in autoimmune hypercholesterolemic mouse model in **Paper 3**. Using two different immunization strategies to target the inflammatory processes of atherosclerosis we aimed to modulate the immunological responses against oxLDL. **Paper 4** adds to this concept by demonstrating that P210-CTB can induce an anti-inflammatory B cell phenotype and reduce atherosclerosis in ApoE$^{-/-}$ mice.

Collectively, the findings in this thesis show that death receptor-associated apoptosis can be linked to cardiovascular outcome in the general population and in patients with systemic autoimmunity. Further, these findings suggest that targeting immune responses against oxLDL with immune-based strategies could represent possible approach for prevention of autoimmune responses in CVD. Atherosclerosis is a complex disease and extensive research about the inflammatory and immune mechanisms is still needed to identify the underlying mechanisms that drives the disease.

Future perspectives

In **Paper 1 and 2**, we provide clinical data of novel biomarkers which might contribute to an earlier identification of patients at risk for CVD in the general population and in individuals with autoimmunity such as SLE. As we have no solid proof other than associations, the next step would naturally be to assess the functional importance of the death receptors in intervention trials. The best scenario to do this, would be to assess patients with manifest atherosclerosis throughout the treatment with a known beneficial medication for atherosclerosis.

We can from **Paper 3** conclude that both immunization strategies are effective in the mouse model used in our experiments and does not induce any apparent adverse effects including aggravation of inflammation. In **Paper 3 and 4** we get a glimpse of possible mechanisms of actions. Regarding which one that is the better option cannot be addressed yet. The methodological approaches and experimental designs

used in our studies all have their individual pros and cons that are important to take into consideration. Thus, more focus on the mechanisms and effects in a human setting needs to be conducted.

Should we treat hyperlipidemia or inflammation?

The work in this thesis focuses on the inflammatory and autoimmune aspects of atherosclerosis. But not to forget, there are other faces of the disease that are equally important in the pathogenesis of the disease. In the cardiovascular community, a question occasionally discussed is if we should focus the therapies in managing hyperlipidaemia or inflammation. It has long been obvious that there are partly conflicting scientific views regarding atherogenesis and it has now roughly emerged into two major approaches; the lipid-centred versus the inflammation-centred view. This is noticeable in several research articles, where the words "lipid driven" or "inflammation driven" often occurs in the objectives of a study. Additionally, other topics such as vascular injury, vessel physiology and genetics are an equally important part of the ongoing investigations in the cardiovascular community but won't be further dealt with in this discussion.

The lipid-centred aspect suggests retention of lipids to be the major contributor to atherogenesis. This was strongly supported by the introduction of lipid-lowering treatments (statins), that had remarkable positive effects in clinical trials.[183] Hence, much research focused on lipids and how they interact with and accumulate in the vessel wall, giving rise to pathological features.[246] The other interpretation favours a fundamental role of the immune system, where pro-inflammatory mechanisms give rise to disease.[213] Thus, the focus has been on inflammatory processes where the role of different immune cell types has been characterized.[247] These processes does not exclude the other and there are strong evidence that the inflammatory reactions arises in response to lipoproteins.[248]

There are paradoxes in the literature regarding management of hyperlipidemia as the underlying cause of CVD. Importantly, statins have been shown to have immunomodulatory effects with an ability to repress MHC-II induction and consequently T cell activation.[222] Not only do these results indicate anti-inflammatory properties with statin treatment, but also the fact that cardiac transplantation patients under statin therapy have a better outcome and survival after the surgery stress the immune effects of statins.[249] Similarly, there are paradoxes regarding inflammation management, where clinical trials with certain anti-inflammatory therapies did not improve clinical outcomes of CVD.[204] The answer to the question "Should we treat hyperlipidemia or inflammation?" cannot be

answered without knowing the true mechanisms of the disease and the relationship between lipids and inflammation.

The cardiovascular community has endless opportunities given by the high number of clinical trials and ongoing research of both lipids and inflammation-driven atherosclerosis. In combination with new emerging techniques and analysis methods, the two sides of the cardiovascular community would benefit from each other's knowledge. This might bring an answer to the mechanisms of the disease allowing the development of better treatments for hyperlipidemia, inflammation or both.

Svensk sammanfattning

Ateroskleros är den vanligaste bakomliggande orsaken till hjärtinfarkt och stroke, som utgör 80% av alla kardiovaskulära komplikationer. Ateroskleros är en kronisk inflammatorisk sjukdom karakteriserad av lipidackumulering, apoptos och fibros i kärlväggen och där immunsystemet spelar en viktig roll i sjukdomsutvecklingen. Immunsystemet har en viktig skyddande funktion i ateroskleros genom att immunceller tar hand om potentiellt skadliga oxiderade/modifierade lipider och apostoliska celler i kärlväggen. Om denna mekanism är otillräcklig kan det leda till kronisk inflammation eller autoimmuna reaktioner. För att förstå hur dessa immunologiska reaktioner är involverade i utveckling av ateroskleros är det därför viktigt att klarlägga orsakerna till uppkomst av autoimmunitet mot komponenter i kärlväggen. I denna avhandling presenteras studier som innefattar immunologiska reaktioner mot ateroskleros-relaterade antigen i människa och mus.

Den första delen av avhandlingen identifierar nya biomarkörer för ateroskleros och deras koppling till specifika kardiovaskulära komplikationer. Vi visar att cirkulerande så kallade dödsreceptorer finns i plasman och att nivån av dessa avspeglar graden av pågående celldöd genom apoptos. Vi visar även att individer med metabola kardiovaskulära riskfaktorer har en förhöjd nivå av cirkulerande dödsreceptorer, vilket talar för att dessa är relaterade till en ökad cellskada. Resultaten visar att individer med tecken på en ökad dödsreceptor-medierad celldöd har ökad risk för att utveckla diabetes och kardiovaskulära sjukdomar. I en annan studie visar vi att plasmanivån av cirkulerande dödsreceptorer även är associerade med kardiovaskulära komplikationer hos patienter med den autoimmuna sjukdomen systemisk lupus erythematosus (SLE). Båda dessa studier ger kliniskt stöd till den så kallade “response-to-injury” hypotesen för hur aterosklerosutveckling sker. Den andra delen av avhandlingen studerar den inflammatoriska komponenten av ateroskleros. Med hjälp av en vaccinformulering som inne håller peptidfragment från apolipoprotein B kan vi visa en möjlig väg för att minska plackutveckling och inflammation i kärlväggen. Vi visar även att antikroppar riktade mot apolipoprotein B peptider har liknande skyddande förmåga. Mekanismerna bakom den skyddande effekten återstår att kartlägga men kan innefatta undanröjningen av oxiderade lipider och inhibering av inflammatoriska reaktioner.

Sammanfattningsvis visar fynden i denna avhandling att dödsreceptor-medierad apoptos kan kopplas till kardiovaskulär risk både generellt och patienter med

autoimmun sjukdom. Vidare talar resultaten för att modulering av immunreaktioner riktade mot oxLDL med hjälp av vaccin eller antikroppsbehandling kan vara möjliga behandlingsformer vid ateroskleros och kanske i synnerhet vid samtidig autoimmun sjukdom.

Acknowledgements

I want to acknowledge those of you who have contributed to this thesis in one way or another. Without the littlest contribution from several people in my environment it would not be a book in your hand at this moment. This acknowledgement is to all of you who have helped me in the lab, guided me in the academy, taught me experimental procedures, proofread my articles or by just being present. Firstly, I would like to acknowledge my supervisors with my deepest gratitude.

Professor Jan Nilsson, my main supervisor. I am grateful for everything you have taught me which is only a fraction of your own knowledge. It amazes me how you can be involved in so many projects ranging from atherosclerosis vaccines to hair research. Also by the fact that you are travelling all over the world but still always able to reach. These past years spent under your supervision have taught me more than I could've imagined and I will forever be thankful for your significant role in my development as a researcher and for offering me this PhD position.

Professor Gunilla Nordin Fredrikson, my co-supervisor. I am so honoured to have had the opportunity to work with you. For allowing me to slow down for a moment to go back one step and reflect. Thank you for always paying attention to small but significant details. I wish you would have gotten a chance to read this thesis with your critical eyes. It has been a privilege to get to know you. **Dr. Maria Wigren**, my co-supervisor. Thank you for teaching me everything in the lab, and always helping out on short notice. Also for practical advices when it comes to experiments and writing. Your great sense of multitasking shows that it is possible to successfully be able to balance up research, family, relations and interests in life.

I want to acknowledge all members of Experimental Cardiovascular Research Unit. **Assoc. professor Eva Bengtsson**, thank you for being the first who introducing atherosclerosis research and for enabling further contacts with the group. I believe you sold the concept of atherosclerosis quite well to me. **Assoc. professor Harry Björkbacka**, thank you for insightful feedback on clinical data and also encouragement to use R.

Dr Sara Rattik and **Dr. Daniel Engelbertsen,** thank you both for sharing your scientific experiences and knowledges. Your practical guidance in manuscript writing, experimental designs and being self-critical of results has given me insights into how to approach my own data. Thanks to **Dr. Pontus Dunér** for being my loyal

cell-line dealer, always able to provide what I need from reagents to fresh strong-brewed coffee. Also for always having the door open for a discussion about science or random things.

Thanks to my fellow PhD's in our group**. Dr. Jenifer Valejo** for always cheering me up with a smile on your face. I'm sure you are enjoying every moment of the sun in San diego now (even at the evenings in the lab). Your determination and hard-working side have shown me that hard work pays off. Soon-to-be **Dr. Lukas Tomas**, thank you for convincing me that flow cytometry is a 'beautifully colourful method', I agree with you now! I'm so impressed by your analytical thinking and I'm sure you will achieve great things both at the clinic and in research. Soon-to-be **Dr. Christoffer Tengryd**, thank you for giving me valuable insights into your clinical CVD research and always taking your time to carefully explain fundamental things I should know about it. Additionally, thank you so much for sharing experiences from your former body-builder career. I'm sorry it did not work out as expected but to be honest I think you have a brighter future as a medical doctor. **Dr. Polyxeni Mantani**, thank you for the philosophical discussions about the future and telling me not to forget to aim for what I truly want and not just first best option. **Dr. Caitriona Grönberg**, thank you for introducing me to the work with clinical data analyses. I wish I had your patience and focus. **Yihong Chen**, welcome to our group, I hope your PhD experience will be amazing.

Importantly, I want to acknowledge the clinical researchers and M.D's in our group for enabling me to see how our research are implemented in the clinic. **Professor Isabel Goncalves** and co-workers. Thank you for being such a warm, caring person. Additionally, you are an excellent researcher and have shown me not to be afraid of standing up for your opinions. **Assoc. professor Alexandru Schiopu** for giving me an insight into how clinical research should be carried out successfully and that it is possible to do it even in different countries simultaneously. **Dr. Andreas Edsfeldt** and co-workers, for your enthusiasm and excitement in receiving new results. Your appreciation makes me realize the importance of every single experiment. Thank you **Dr. Goran Marinkovic** for teaching me your myocardial infarction model and always lending me antibodies in the last minute. Also for allowing me to practice blood sampling, I have no idea how you could even let me do that to you?!

A big thank you to all my lab-mums, whom I have gotten the honour of having my office together with. Even though you talk a lot and even had fika 45 minutes once, the days would not be as fun without you. I know that now, because I had to write my thesis away from all of you which was really boring. **Lena Sundius**, thank you for bringing me perfectly made coffee (cardboard coloured, very important), providing me sugar when I'm falling asleep and just daily pranks in general. But not to forget, thank you for all the hours together spent dissecting tissue with **Irena Ljungcrantz**. I don't know how I would have managed my *in vivo* work without

the help from you. Especially as I'm the slowest in dissecting and makes the ugliest *en face* flat preps of us three, it proves that this thesis might not even exist without your help.

Also thank you **Linda Andersson**, **Fong To** and **Mihaela Nitulescu** for always being able to help out in the lab when it's a crisis, reminding me its lunch-time and stresses the importance of daily walks or gympa that I never attend anyways. Thanks to **Ana Persson** for always being ready with your nurse kit and Napolitanke and **Gertrud Jensen** for keeping all the crazy hens in the cage. **Anna Maria Dutius**, thank you for sharing my interests about kidney histology, true crime and horse hoof-tentacles.

Fellow PhD and research friends; **Sabrina Hsiung, Azra Alajbegovic, Katarzyna Krawzyk** and **Dr. Anki Knutsson**. Thank you for sharing frustration, success and most importantly support throughout this journey. **Anahita Abdali** and **Emelie Larsson** for encouragement and coffee breaks during my first PhD year. Also thanks to my students for giving me the experience to supervise. **Oscar van der Have**, I must admit we spent too much time looking at gross medical mystery-clips and surgeries…

I would further like to acknowledge important collaborators. **Professor Anders A Bengtsson**, for opportunities to explore atherosclerosis and autoimmunity with the SLE samples. To **Professor Anna Hultgårdh-Nilsson**, who have given me opportunities to share my knowledge in her lab and letting me have an office space when needed. Thanks to the **VIA consortium** for educational meetings; **Cardiovax** for interesting discussions about atherosclerosis, immunology and cancer research. **Professor Jan Holmgren** and co-workers for providing us with peptides used in some of my projects.

I also want to acknowledge the **Wallenberg Centre for molecular medicine** Fellowship of 2018-2019, both **students and PI's**. I am grateful for all the knowledge provided from each other's research fields. Especially thanks to **Assoc. professor Anja Meissner**, for your mentoring. Your enthusiasm about research, determination to achieve what you want and competence in our field proves what it takes to succeed in academia.

Additionally, I want to acknowledge those who motivated me to pursue research that lead to starting a PhD. Thanks to the group of **Vascular Physiology** for giving me the opportunity to conduct not only one, but three student projects. Also for the opportunities to learn how to properly do cell culturing and work with live vessel systems. Thanks to **Redoxis** for giving me knowledge about a variety of disease models as well as excelling my *in vivo* methodology to such an advanced level.

I would also like to acknowledge **Professor Rikard Holmdahl**, for being the first who introduced research to me at all. Thanks for the possibilities at **Medical**

Inflammation Research, where I was given valuable skills in handling animals and insights into experimental models and later **MBB** at Karolinska Institute for giving me my first responsibilities *in vivo*. Especially **Carlos** and **Kristina Palestro** who made summer work great fun. Thanks to **Assoc. Professor Thomas Blom** for introducing me to autoimmunity.

Lastly but absolutely not the least, I would like to acknowledge a significantly important group of people outside of the scientific community; namely my **family** that are spread all over the world and all of my **friends**.

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