From the Department of Medicine
Karolinska Institutet, Stockholm, Sweden

TUMOR NECROSIS FACTOR SUPERFAMILY MEMBERS CD137 AND OX40 LIGAND IN VASCULAR INFLAMMATION

Leif Å Söderström, MD

Karolinska Institutet

Stockholm 2016

Published by Karolinska Institutet.
Printed by E-Print AB 2016

ISBN 978-91-7676-187-8

“The more I practice, the luckier I get” –Gary Player

To my family

TUMOR NECROSIS FACTOR SUPERFAMILY MEMBERS CD137 AND OX40 LIGAND IN VASCULAR INFLAMMATION
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Leif Å Söderström, MD

Principal Supervisor:
Dr Peder S Olofsson
Karolinska Institutet
Department of Medicine
Division of Cardiovascular Medicine Unit

Co-supervisors:
Professor Göran K Hansson
Karolinska Institutet
Department of Medicine
Division of Cardiovascular Medicine Unit

Dr Maria L Klement
Karolinska Institutet
Department of Medicine
Division of Cardiovascular Medicine Unit

Opponent:
Professor Andreas Zirlik
University of Freiburg
Department of Medicine
Division of Cardiology and Angiology

Examination Board:
Professor Michelle Chew
Linköping University
Department of Medical and Health Sciences (IMH)
Division of Drug Research

Professor Ralph Knöll
Karolinska Institutet
Department of Medicine, Huddinge (MedH), H7
Division of Integrated Cardio Metabolic Centre

Associate Professor Lisa Westerberg
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology

ABSTRACT

Atherosclerosis, an inflammatory disease, is the major cause of cardiovascular disease - the main cause of death worldwide. T cells are central orchestrators of inflammation in atherosclerosis and critically depend on costimulation for adequate function. Hence, costimulation is pivotal for maintaining immunological homeostasis of inflammatory responses, and a dysregulated immune response may aggravate inflammation in atherosclerosis. Costimulators are therefore of central interest in the pathogenesis of cardiovascular disease. CD137 and OX40 ligand are important costimulatory molecules of the tumor necrosis factor superfamily, but their role in vascular inflammation has been unclear.

We used human biobanks and clinical cohorts in combination with experimental models of atherosclerosis and atherothrombosis to investigate the involvement of CD137 and OX40 ligand in the pathogenesis of cardiovascular disease.

We observed that CD137 was expressed in human and murine atherosclerosis, and that activation of CD137 promotes inflammation and atherosclerosis development in hypercholesterolemic mice. By studying gene expression in cell lines, we found an association between the single nucleotide polymorphism (SNP) rs2453021 and CD137 mRNA expression in human lymphoid cells. The minor allele of this SNP was associated with an increased intima media thickness in human carotid arteries in individuals with risk factors of cardiovascular disease. To study the influence of CD137 activation on atherothrombosis, we turned to an experimental plaque rupture model. We observed that CD137 mRNA expression was higher in ruptured compared to non-ruptured murine carotid lesions. Stimulation of CD137 promoted vascular and systemic inflammation, but did not increase plaque rupture frequency.

Others have reported an association between the SNP rs3850641 in OX40 ligand and cardiovascular risk. We did observe expression of OX40 ligand on endothelial cells within human carotid atherosclerotic lesions, and the OX40 ligand expression was induced by tumor necrosis factor (TNF) in cultured vascular endothelial cells. However, we found no association with the risk for stroke in two independent populations.

In conclusion, the studies in this thesis demonstrate expression of CD137 and OX40 ligand in human atherosclerotic lesions, and that activation of CD137 promotes inflammation and atherosclerosis development in hypercholesterolemic mice. These new insights on the pathophysiology of atherosclerosis warrant further studies of the therapeutic potential of interventions in costimulation for treatment of cardiovascular disease.

LIST OF SCIENTIFIC PAPERS

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PMID: 18285570

II. Genetic variants of TNFSF4 and risk for carotid artery disease and stroke.
Olofsson PS, **Söderström LÅ**, Jern C, Sirsjö A, Ria M, Sundler E, de Faire U, Wiklund PG, Ohrvik J, Hedin U, Paulsson-Berne G, Hamsten A, Eriksson P, Hansson GK. J Mol Med (Berl). 2009 Apr;87(4):337-46. doi: 10.1007/s00109-008-0412-5. Epub 2008 Nov 8.
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IV. Effects of the CD137-activating antibody 2A in a murine inducible plaque rupture model
Söderström LÅ, Jin H, Klement ML, Gisterå A, Perisic L, Hedin U, Paulsson-Berne G, Maegdefessel L, Hansson GK and Olofsson PS. Manuscript.

CONTENTS

LIST OF ABBREVIATIONS

ACS	Acute coronary syndrome
AP	Angina pectoris
AP-1	Activating protein 1
APC	Antigen presenting cell
ApoE	Apolipoprotein E
CAD	Coronary artery disease
CCL-2	Chemokine (C-C motif) ligand 2
CD	Cluster of differentiation
CIA	Collagen induced arthritis
CRP	C-reactive protein
CTLA4	Cytotoxic T-lymphocyte-associated antigen 4
CVD	Cardiovascular disease
DAMP	Danger associated molecular pattern
DC	Dendritic cell
DNA	Deoxyribonucleic acid
EAE	Experimental autoimmune encephalitis
EC	Endothelial cell
Foxp3	Forkhead box P3
HDL	High-density lipoprotein
ICAM-1	Intracellular adhesion molecule 1
IFNγ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IMT	Intima media thickness
kDa	kilo Dalton
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
M-CSF	Macrophage colony stimulating factor
MAPK	Mitogen-activated protein kinases

MCP-1	Monocyte chemoattractant protein 1
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
NFκB	Nuclear factor κ-light- chain enhancer of activated B cells
NIK	NFkB inducing kinase
NK cells	Natural killer cells
NKT cells	Natural killer T cells
NSTEMI	Non-ST-elevation myocardial infarction
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RANKL	Receptor Activator of Nuclear Factor Kappa B Ligand
SLE	Systemic lupus erythematous
SMC	Smooth muscle cell
SNP	Single nucleotide polymorphism
STEMI	ST-elevation myocardial infarction
TCR	T cell receptor
TGFβ	Transforming growth factor beta
TIA	Transitory ischemic attack
TLO	Tertiary lymphoid organ
TLR	Toll-like receptors
TNF	Tumor necrosis factor
TNFRSF	Tumor necrosis factor receptor superfamily
TNFSF	Tumor necrosis factor superfamily (ligand)
TRAF	TNF receptor associated factor
Treg	Regulatory T cell
UA	Unstable angina
VCAM-1	Vascular cellular adhesion molecule 1
VSMC	Vascular smooth muscle cell

1 INTRODUCTION – A NEED FOR NEW DISCOVERIES!

Inventions and development are critically dependent on new discoveries and, perhaps sometimes, novel ways of looking at old discoveries. By using the enormous amounts of existing knowledge, we can use new technical tools to look at old problems with better resolution, or develop new concepts that makes us view problems in a completely novel way. Pre-clinical laboratory research is frequently done in a milieu far away from the patients struck by an acute life-threatening disease. In the clinic, far from the laboratory, we still lack adequate methods to predict, identify, and treat patients with the disease that is the most common cause of death globally – cardiovascular disease. Even though the patients receive state of the art medical treatments, and get the best possible care by dedicated doctors and nurses, the suffering of patients and the persons close to the affected patients is still immense. To get maximum benefit from the joint efforts and expertise, the need for a mutual interface between the experimental and clinical branches of cardiovascular research becomes more evident as the complexity of atherosclerosis unravels. Since the underlying mechanisms of cardiovascular disease are not fully known, the endeavor for cure and prevention is yet to be completed, and the road ahead is doubtlessly bordered by novel discoveries.

2 AIMS

The overall aim of this thesis was to determine the roles of the costimulatory molecules CD137 and OX40 ligand in atherosclerosis and cardiovascular disease.

The specific aims were to:

- investigate CD137 expression in atherosclerosis and determine whether activation of CD137 aggravates disease
- investigate genetic influence on human CD137 expression and its association with cardiovascular disease
- determine whether CD137 activation promotes rupture of atherosclerotic plaques
- describe OX40 ligand expression in human atherosclerotic lesions and determine whether the minor allele of rs3850641 in *OX40 ligand* is associated with increased risk for stroke

3 A COSTIMULATORY PERSPECTIVE ON THE IMMUNE SYSTEM

The immune system is vital for the survival of all human beings. A constant battle against external and internal pathogens is normal. The battle is fought by immune cells and is regulated by receptors that detect danger, by cytokines, cell-cell interactions, neural reflexes and more. Immune cells with well-defined specificities and functions interact to optimize homeostasis and promote health. Often, macrophages and dendritic cells (DC) phagocytose infected and damaged tissue, and present fragments i.e. antigens, to the central orchestrators of immune responses, T cells. If a T cell recognizes a dangerous fragment, for example a piece of a known microbe, it does not run ahead to immediate war. Instead, it stops and awaits a "go-signal" or a "stop-signal" from the immune cell presenting the antigen. This is a key feature of the immune response that protects from senseless over-activation of the very powerful T cells, and ensures that the response to threat will be adequately measured. These control signals are called "costimulatory" or "coinhibitory" and are performed by so called costimulatory and coinhibitory receptors and ligands (Figure 1). The costimulatory molecules have emerged as key players in an adequate functional immune response. Their importance is underscored by their functionality as pivotal on- or off-buttons for the cellular immune response. In addition, costimulation is essential for fine-tuning of the inflammatory response [1, 2] and has been widely studied in the context of the acquired, so called adaptive, immune response [3].

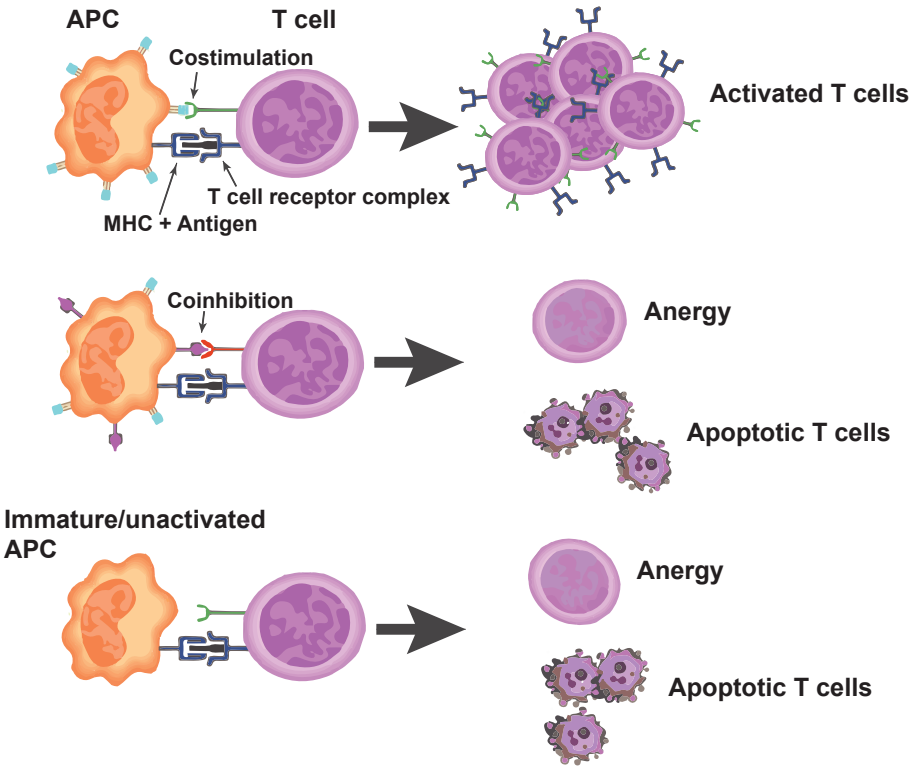

Figure 1. Costimulation and coinhibition of T cells. Both T cell antigen recognition and costimulation is needed for activation of T cells. Coinhibition or lack of costimulation promote anergy or apoptosis of the T cell. Definitions are in the list of abbreviations.

The most well-characterized costimulatory and coinhibitory molecules are of the CD28/B7 family, and the costimulatory molecules of the tumor necrosis factor superfamily (TNFSF), which include CD137, CD137 ligand (CD137L), OX40 and, OX40 ligand (OX40L).

The CD28/B7 pathway is prototypical for costimulation. CD28 is expressed on T cells and is the receptor for the classical "signal 2" which provides the "go ahead" for T cells that have recognized an antigen ("signal 1"). The B7 family molecules B7-1 (CD80) and B7-2 (CD86) ligate CD28 resulting in costimulation. The B7-1 and B7-2, may also ligate the cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) (CD152) providing an inhibitory signal balancing the response.

3.1 COSTIMULATION OF T CELLS

T cells recognize antigens presented on major histocompatibility complex (MHC) molecules by specialized antigen presenting cells (APC), including macrophages, DC and B cells. T cells occur in a number of varieties, including $CD4^+$ helper T cells, which also support B cell function, and $CD8^+$ cytotoxic T cells, which kill infected or transformed cells [3]. $CD4^+$ T helper cells recognize antigens presented on MHC class II molecules by APCs. The adaptive response is fine-tuned by costimulatory signals, and lack of costimulation in T cells results in T cell anergy or apoptosis [4] (Figure 1). T cell expression of the costimulatory molecule CD137 is not ubiquitous, but occurs on subgroups of $CD4^+$ T cells and on $CD8^+$ T cells, and is mostly associated with activation [1] (see also Table 1). Hence, presence or absence of CD137 and its ligand can determine the fate, anergy/apoptosis or activation/proliferation, of particular T cells in an immune response. Furthermore, effects of costimulation may also differ between effector T cell subsets. This is exemplified by CD137 that has a preference for controlling $CD8^+$ T cell response [5, 6].

Regulatory T cells (Treg) include the $CD4^+CD25^+Foxp3^+$ subpopulation. Treg are essential for maintaining immunological homeostasis and for controlling immunological tolerance. Mice deficient in Treg develop an autoimmune phenotype [7]. Costimulatory factors play a key role in Treg biology. For example, ligation of CD137 expressed on Treg results in proliferation, which may suppress immune responses [8, 9]. Interestingly, ligation of OX40 by OX40L can inhibit the conversion of naïve T cells into $Foxp3^+$ Treg, and suppress the TGFβ driven $Foxp3^+$ development [10]. Hence, costimulatory molecules of the TNFSF can have opposite effects on Treg. Thus, costimulation of T cells through members of the TNFRSF can either promote or inhibit Treg development, and fine-tune an ongoing inflammatory response in intricate ways that as of yet are only partly understood.

Coinhibitory signals are negative regulators of T cell responses and coinhibitory receptors partly share ligands with the costimulatory receptors. Hence, the timing, balance and spatial distribution of costimulatory and coinhibitory receptor and ligand expression can exert pivotal effects on T cell activation and on important aspects of the immune response.

3.2 COSTIMULATION BEYOND T CELLS

Interestingly, there are a growing number of reports that support a vital role for costimulatory molecules also in the inborn, so called innate, immune response. For example, CD137 deficient mice are resistant to endotoxemic shock, a model of severe, acute septicemia [11]. Moreover, CD137 deficient mice have fewer natural killer (NK) cells, key cells for the innate defense against viral infection and tumor formation. In fact, most cells of the innate immunity, such as macrophages, express and respond to signals through costimulatory and coinhibitory molecules [3]. Furthermore, OX40L, expressed on APCs and ECs play a role in the neo-formation of microvessels in atherosclerotic lesions, a process involving several cell types [12]. Cells known to express the TNFSF members CD137, CD137L, and OX40L are listed in table 2.

3.3 A STIMULATORY CONCLUSION

Considering the crucial importance of costimulation in adaptive and innate immunological homeostasis, it is not surprising that manipulation of costimulatory molecule activity has a significant impact in a number of experimental inflammatory diseases. For example, in models of systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and atherosclerosis, manipulation of costimulation lead to profound effects on inflammation and disease [12-14]. Both the innate and the adaptive immune systems are essential for host defense and for tissue repair, and it has become more evident that the cross talk between innate and adaptive immunity is a crucial function for the immune system as a whole. Costimulation is pivotal for maintaining immunological homeostasis, and a dysregulated immune response may result inflammatory diseases, among them atherosclerosis.

Table 1. T cell subsets relevant for CD137

	Inducing cytokines	Typical cytokines	Effect in atherosclerosis	Effect of CD137 ligation	Reference
Helper T cells ($CD4^+$)					
Th1	IFNγ, IL-12	IFNγ, TNF, IL-2	Pro-inflammatory	Activation, proliferation, prolonged survival	Szabo *et al.* 2000[15]; Mosman *et al.* 1986[16]
Th2	IL-4, IL-33	IL-4, IL-5, IL-13	Unclear	Activation, proliferation, prolonged survival	Zheng *et al.* 1997[17]
Th17	IL-6, TGFβ	IL-17	Unclear (pro-atherosclerotic and athero-protective)	Inhibits generation of Th17 cells	Ivanov *et al.* 2006[18]; Veldhoen *et al.* 2006[19]; Kim Choi *et al.* 2011[20]; Gisterå *et al.* 2013[21]
Treg	IL-2, TGFβ	TGFβ, IL-10, IL-35	Athero-protective	Proliferation, more	de Boer *et al.* 2007[22]; Klingenberg *et al.* 2013[23]; Mallat *et al.* 2003[24]
Cytotoxic T cells ($CD8^+$)	-	IFNγ, TNF, IL-2, more	Stim of CD137 increases $CD8^+$ infiltration	Activation, proliferation, prolonged survival	Gotsman *et al.* 2007[25]; Bu *et al.* 2011[26]; Watts 2005[2]

Definitions are in the introductory list of abbreviations.

4 ATHEROSCLEROSIS

4.1 ON THE AGE OF ATHEROSCLEROSIS

Atherosclerosis is a smoldering chronic inflammatory disease that develops over decades. For thousands of years, atherosclerotic disease has affected large and medium size arteries. Findings in ancient Egyptian mummies, and in mummies from Alaska, reveal atherosclerotic lesions similar to lesions of the modern human being [27-31]. This suggests that atherosclerosis not only depends on modern lifestyle, but also has a complex pathophysiology involving ancient physiological mechanisms, such as inflammation.

Inflammation was introduced 25 AD by Celsus who described the features of an inflammatory reaction as: rubor (redness), calor (heat), dolor (pain), and tumor (swelling) [32]. Later, another feature, Functio laesa, was added to the other four describing the loss of normal function of the inflamed tissue [33]. In the middle of the 19th century, Rudolf Virchow described the cellular pathology of the atherosclerotic lesions and claimed that inflammatory cells play a role in the pathophysiology [34]. Adding to the findings of Virchow more than a century ago, Nikolaj N. Anitschkow and Semen S. Chalatov published their experimental finding that a cholesterol rich fed to rabbits led to atherosclerosis [35]. Later, Anitschkow showed that atherosclerotic plaques consist not only of lipids but also inflammatory cells, including lymphocytes and macrophages [36] - the same observation as Virchow had done almost half a century earlier. The discovery of immune cells in atherosclerosis did not attain the same historical distinction as cholesterol, and atherosclerosis was later considered a product of hyperlipidemia, platelet accumulation, and shear stress, leading to endothelial damage, deposition of lipids, and smooth muscle cell proliferation [37]. Using immunohistochemical methods with monoclonal antibodies, Jonasson *et al.* observed activated immune cells within the atherosclerotic lesion in the mid 1980's. They were able to clarify the cellular composition of the atherosclerotic plaque and expose the presence of macrophages and activated T cells. Their published report is a hallmark for modern atherosclerosis research [38].

When considering atherosclerosis of the arterial wall in light of present evidence, there is an orchestrated interplay between different immune cells within the innate and adaptive immune system, and other cells that are immunologically active, but that traditionally not belong to the immune system, e.g. endothelial cells (EC) and smooth muscle cells (SMC) in the progressing lesion [39]. The subsequent studies of immunological mechanisms in atherosclerosis have revealed an intricate complexity in the pathogenesis in the atherosclerotic lesion. For example, the discoveries of toll-like receptors (TLR) in atherosclerosis [40] and different subsets of T- and B lymphocytes have deepened the knowledge on the mechanisms causing this chronic inflammation in the arterial wall [39].

There is ample evidence that the inflammatory activity is a key player in lesion development as well as in plaque rupture - the event responsible for most clinical manifestations of

cardiovascular disease [39, 41-46]. The development of atherosclerosis and its clinical manifestations is clearly a complex process that involves a sophisticated interplay between inherited host factors, lipid metabolism, inflammation, circulation physiology, coagulation, and probably a host of additional yet not fully recognized factors.

4.2 ON THE PATHOPHYSIOLOGY OF ATHEROSCLEROSIS

A normal healthy artery consists of three distinct layers (Figure 2): 1) the tunica intima with an endothelial monolayer resting on the basal membrane, and a few resident smooth muscle cells; 2) the tunica media composed of SMC and extra-cellular matrix, separated from the intima by the internal elastic lamina; and 3) the tunica adventitia containing loose connective tissue, mast cells, nerve endings and micro vessels, separated from the media by the external elastic lamina, [47].

4.2.1 Onset of atherosclerosis

Atherosclerotic lesions start to develop early in life. Beginning as a fatty streak in the intimal layer of the arterial wall, low-density lipoprotein (LDL) containing cholesterol that has entered the subendothelial space of the vascular wall triggers an inflammatory response. The cellular components of the early fatty streak are macrophages, T cells, and mast cells, the latter two less numerous than macrophages [44] (Figure 2). *Post-mortem* investigations of prevalence of fatty streaks in 2 to 39 years old individuals have shown that practically all included subjects had fatty streaks in the aorta, and that approximately 50% of subjects aged between 2 to 15 years had fatty streaks in the coronary arteries. Eight percent of the investigated subjects at 2 to 15 years of age had plaques in the coronary arteries. The prevalence increased with age to 69% in subjects 26 to 39 years of age [48]. Previous studies of soldiers killed in battle during the Korean war show a similar pattern, with a prevalence of about three-quarters of soldiers with an average age of 22 years where evidence of coronary atherosclerotic disease was found. Of these, 15% had a luminal narrowing in one or more vessels ranging from 50% to complete occlusion [49]. Taken together, these studies show that a majority of the study participants had manifest atherosclerotic disease, and that virtually all had the early stages of atherosclerosis that could develop into advanced disease.

4.2.2 Development of the atherosclerotic plaque

An activated endothelium will express adhesion molecules, an important factor for atherosclerosis formation. These molecules recruit leukocytes to the developing lesion. Together with lipid depositions in the arterial wall, the recruited leukocytes start the development of an atherosclerotic plaque. Anatomical features, such as curvatures and branches of the arterial tree, form the basis of flow and different levels of shear stress on the endothelium. Reduced shear stress may lead to activation of the endothelium, [50, 51].

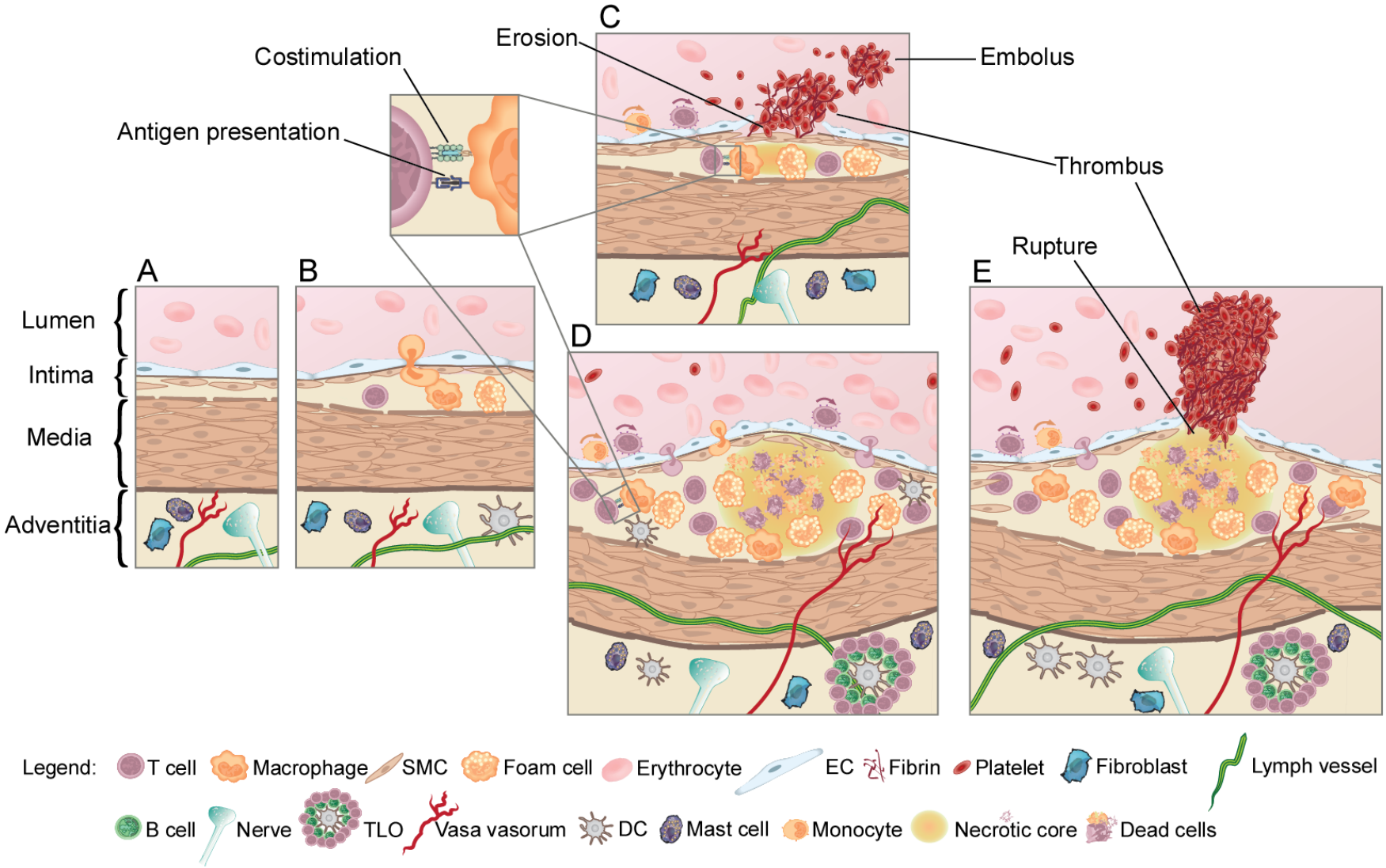

Figure 2. Development of a symptomatic atherosclerotic lesion. A) Normal artery, B) Initiation of the atherosclerotic lesion, C) Plaque erosion, D) Advanced lesion, E) Plaque rupture. Definitions are in the list of abbreviations.

The dominant cell type recruited is monocytes that will differentiate into macrophages. Some macrophages will engulf modified cholesterol particles such as oxidized LDL, eventually developing into foam cells. Activated macrophages produce pro-inflammatory cytokines such as tumor necrosis factor (TNF), and interleukin-1β (IL-1β), thus contributing to the pro-inflammatory milieu in the developing atheroma [39]. Neutrophils are present at the early stages of lesion development, and have been suggested to play a role at sites of plaque erosion [52]. T cells and macrophages, and a few other inflammatory cells, such as mast cells and DC, accumulate in the forming atherosclerotic lesion. T cells play a central role in regulating the inflammatory milieu [39] (Figure 2). No single pivotal factor that continuously drives this non-resolving inflammation has been identified, but several factors have been proposed, e.g. endogenous parts of LDL [39, 53] and crystalized cholesterol [54]. The inflammatory activity in the lesion may result in production of cytokines and chemokines, such as IL-6 and monocyte chemoattractant protein 1 (MCP-1), and as a consequence, the recruitment of immune cells [55]. Subsequently, IL-6 production may lead to the synthesis and release of C-reactive protein (CRP) from the liver. Elevated CRP has been recognized as an independent risk factor for atherosclerosis [46].

SMC produce collagen, which contributes to plaque stability. SMCs migrate from the tunica media, and SMCs from the intima proliferate, both contributing to the build-up of a fibrous cap enclosing the forming lesion. Beneath the fibrous cap, some of the cells die leaving debris and cholesterol that form the highly thrombogenic necrotic core. Large advanced lesions may also be supported by new microvessels, which facilitate a communication between the developing lesion and the patrolling immune cells [47] (Figure 2).

4.2.3 The role of tunica media and tunica adventitia in atherosclerosis

Most atherosclerosis research has focused on the intima and the formation of an atherosclerotic lesion within intima. However, the role of tunica media and tunica adventitia has gained increased attention. For example, vascular smooth muscle cells (VSMCs), the most abundant cell type of the tunica media, are capable of expressing TLRs [56] and are able to produce inflammatory cytokines such as IL-1, IL-6, and TGFβ [57]. Interestingly, the tunica media is sparsely infiltrated with leukocytes, suggesting an immunoprivileged site restricting the neointimal inflammation from spreading to the medial layers [58]. The outermost layer of the vessel is the tunica adventitia, which contains mast cells, DCs, lymphatic vessels, nerve endings, and late in the process tertiary lymphoid organs (TLO) [47]. Lymphatic vessels drain to lymph nodes, which provide a path for immune cells, e.g. APCs, to migrate to lymph nodes and present antigens from the lesion. This creates a foundation for immune responses towards antigens generated in the atherosclerotic lesion [47]. The atherosclerotic microvessels probably come from the adventitia, stretching into the inner layers of the vessel providing connections between the deep layers of the lesion and the circulation [47, 59]. TLOs develop at sites of non-resolving inflammation, and can be located in the tunica adventitia adjacent to atherosclerotic lesions. In the TLOs of the adventitia,

T cells and B cells interact with APC, and germinal centers are formed, emphasizing the potential role of TLO in lesion development [60].

Taken together, available evidence points towards an interaction between all layers of the atherosclerotic artery as an important part of pathophysiology [47, 57].

4.2.4 Lipids

LDL levels in blood correlate with the risk for cardiovascular disease, and the associated risk is synergistically amplified when increased in combination with elevated CRP [46]. The levels of LDL can be lowered in patients with treatment with hydroxymethyl coenzyme A reductase inhibitors (statins), and patients treated with statins have lower risk of cardiovascular events [61-63]. Although beneficial, statin treatment is not a sufficient treatment for atherosclerosis. The risk of adverse cardiovascular events in patients with advanced atherosclerotic lesions is still present, in spite of statin treatment [45]. However, widespread statin therapy have changed not only lipid profile, but may also have affected inflammatory features of the plaque, such as reduced macrophage content in carotid lesions [64-66]. In addition, the distribution of clinical manifestations of atherosclerosis may have shifted somewhat, but the cause of these changes is not fully known (see also section 4.2.5) [43].

High-density lipoproteins (HDL) are sometimes referred to as “the good cholesterol”. Indeed, low plasma levels of HDL have been recognized as a risk factor for cardiovascular disease (CVD) [67]. HDL particles can act as transporter proteins for cholesterol from the peripheral tissues. By unloading cholesterol from foam cells in the atherosclerotic lesion and transport it to the liver, HDL can decrease the lipid content in the atheroma. Furthermore, separately from its lipid-transporting role, HDL may also reduce inflammation [68]. In contrast, clinical trials targeting HDL has not shown significant benefits [61]. Thus, whether the decreased cardiovascular risk associated with increased HDL is related to a decreased lipid burden of the atheroma or reduced inflammation is yet unclear.

4.2.5 Plaque rupture and plaque erosion

Post-mortem studies show that about 70% of the fatal myocardial infarctions are due to plaque rupture with an intraluminal thrombus limiting blood flow [45, 69]. The remaining 30% are assumed to be caused by plaque erosion [39, 45, 70]. Plaque rupture is the main cause of coronary thrombosis, and is more common in men than in women [71].

Plaque rupture is defined as a structural defect in the fibrous cap of the atherosclerotic lesion separating the lipid-rich necrotic core from the blood stream [72]. The rupture will expose thrombogenic substances, such as tissue factor and lipids, to the blood stream. This will lead to a thrombus formation and subsequent impairment of the blood flow, either at the site of the rupture, or downstream in the vasculature by embolism [45, 73] (Figure 2). Atherosclerotic plaques associated with rupture are rich in lipids, poor in collagen, have thin fibrous caps, and

have abundant inflammation [43]. The exact mechanism triggering the rupture is not known, but intense inflammatory activity has been suggested [73].

Plaque erosion is defined as a plaque with loss of luminal endothelial cells with no structural defect is seen in the fibrous cap. The loss of endothelium exposes SMCs and proteoglycans to the blood stream leading to a thrombus formation. In addition, a plaque with endothelial dysfunction that leads to a thrombus formation is also defined as an eroded plaque, even if the ECs have not disappeared [72]. Plaques associated with erosion have different features compared to plaques associated with rupture. For example, plaques associated with erosion are rich in proteoglycans and glycosaminoglycans, have fewer inflammatory cells, and lack large lipid pools [70].

In carotid atherosclerosis, the ratio between rupture and erosion as the cause of symptoms is similar to the ratio in coronary arteries [74]. However, in patients presenting with transitory ischemic attack (TIA), the ratio of erosion is higher [75]. Furthermore, in women under 50 years of age that suffered from sudden coronary death, the rupture rate is lower in favor of erosion [45, 71]. Given the trajectory of morphological changes over the last decades [65], endothelial erosion may become an even more prominent issue in the future.

Interestingly, recent evidence indicates that plaques with thin caps and high lipid content rarely rupture to cause clinical events [76]. This suggests that clinical disease is not caused by a single lesion that is easily identifiable prior to clinical symptoms, and that there are several plaques capable of causing clinical disease [77, 78]. This notion was supported by a study using intravascular ultrasound where approximately 5% of plaques with thin caps ruptured during a follow-up period of 3.4 years [76].

One study of carotid atherosclerosis shows a morphological shift towards smaller lesions in recent time [65]. Van Lameren *et al.* reported that not only did the plaque size decrease, but there was also a decreased frequency of plaques with large lipid cores, and high macrophage content. Furthermore, the occurrence of plaque thrombosis and degree of plaque calcification also decreased. High macrophage content and large lipid pools are characteristics of complex atheroma, and the decline of these features are consistent with observed decline in the incidence of ST elevation myocardial infarction (STEMI) vs. non-ST elevation myocardial infarction (NSTEMI) ratio [65], and the observed decline in stroke [79, 80]. These observations indicate a decrease in plaques prone to rupture, and a relative increase of plaque erosions [43]. Of note, erosion is more common in women, in patients with diabetes, and in elderly patients, and this coincides with the observed demographic changes in patients with acute coronary syndrome (ACS) [43]. It has been suggested that these changes in plaque composition over time, and possibly the pathophysiology of atherothrombotic disease, has been driven by the use of statins [43], but several other factors also need to be considered, for example changes is smoking and dietary habits [81].

The introduction of statins together with new ways of examination, such as intraluminal ultrasound, has revealed both a new face of the disease, and new ways of evaluating disease

in patients. Bearing this in mind, however, one should not forget that plaque rupture still accounts for a vast majority of fatal myocardial infarctions and inflammation intensity is still of great importance. Thus, the change in atherosclerotic features adds to the complexity of the disease and demands a new research approach, including not only the classical features of plaque rupture, but also methods detecting erosion as a mechanism of atherothrombosis.

4.2.6 Inflammation and plaque physiology

Abundant evidence indicates that local inflammation in the vascular wall plays an important role for precipitation of clinical events, plaque rupture, erosion, and thrombus formation.

Immune responses are commonly initiated by signals in pattern recognition receptors, including TLRs, nucleotide-binding oligomerization domain-like receptors (NLRs), and others. Pattern recognition receptors are expressed within atherosclerotic plaques [40]. Interestingly, atherosclerotic ECs express TLR2 that may recognize pathogen associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs). TLR2 is primarily expressed in SMC-rich plaques and receptor binding may promote EC activation and/or apoptosis of the EC, processes that may lead to detachment of the EC and subsequent exposure of thrombogenic substances to the blood stream [52]. Other mechanisms influencing EC adherence, such as mechanical stimuli promoting vascular SMC production of proteoglycans and hyaluronan [82], may act in concert or independent of TLR2 [70].

Increased local levels of repair-associated cytokines such as TGFβ stimulate SMC to produce collagen that contributes to mechanical cap strength [83, 84]. Conversely, plaque inflammation leads to increased local levels of IFNγ, which inhibits SMC proliferation and collagen production and degradation [84-86].

4.2.6.1 T cells in atherosclerosis

Several subtypes of T cells have been implicated in atherosclerosis (Table 1). T cells are an essential part of the atherosclerotic disease, and the interaction between several cells, including different T cell subsets, B cells, and innate immune cells, within the atherosclerotic lesion decides the fate of the atherosclerotic lesion [39].

Since the inflammatory response in atherosclerosis is orchestrated by T cell activity, the costimulation necessary for activation of T cells is important. Several costimulatory molecules have been implicated in atherosclerosis. For example, CD40L deficiency reduces atherosclerosis in apolipoprotein E deficient (*Apoe*$^{-/-}$) mice [87], and OX40L deficiency in *Apoe*$^{-/-}$ also reduces atherosclerosis [12]. Interestingly, CD40 deficiency in *Apoe*$^{-/-}$ mice reduced atherosclerosis [87], but not in low-density lipoprotein (LDL) receptor deficient (*Ldlr*$^{-/-}$) mice [88], indicating a complex function for costimulatory molecules in atherosclerosis.

4.2.6.1.1 T helper 1 cells

T helper 1 (Th1) cells are $CD4^+$ and are present in atherosclerosis [39]. The signature cytokine of Th1 T cells is IFNγ. In mice, IFNγ promotes atherosclerosis, and this is also shown in human arteries transplanted into mice [73, 89]. Conversely, atherosclerosis in *Ifnγ*$^{-/-}$ mice is reduced compared to *Ifnγ*$^{+/+}$ control mice [90]. Several cells within the atherosclerotic lesion are capable of producing IFNγ, including macrophages [73] and natural killer T (NKT) cells [91]. IFNγ serves as one signal of the classical macrophage activation [92]. In addition, IFNγ promotes upregulation of MHC class II, and inhibits SMC proliferation and production of collagen [84, 85], features that has been associated with plaques rupture [93].

4.2.6.1.2 T helper 2 cells

$CD4^+$ T helper 2 cells are present in human and murine atherosclerosis but the frequency compared to other T cell subsets is unclear [39, 94]. Th2 cells produce cytokines such as IL-4, IL-5, IL-10, and IL-13. Several of the Th2 cytokines have been suggested to be atheroprotective. For example IL-10, a cytokine also produced by Treg, reduces atherosclerosis [95] as does IL-5 [96]. In contrast, deficiency of the Th2 signature cytokine IL-4 has been shown to decrease atherosclerosis [97, 98]. Therefore, the net effect of Th2 activation on atherosclerosis is unclear.

4.2.6.1.3 Cytotoxic T cells

Cytotoxic $CD8^+$ T cells usually occur in low numbers in experimental murine atherosclerosis [41]. In human atherosclerosis, $CD3^+$ lymphocytes account for about 10% of cells and $CD8^+$ T cells account for about a third of the $CD3^+$ cells, a proportion similar to peripheral blood [73]. Interestingly, a recent study by van Dijk *et al.* show a dynamic pattern with higher $CD8^+/CD4^+$ ratio in early stages of human atherosclerosis, a ratio that will be almost equalized in later stages [94]. Cytotoxic T cells exert their effect by inducing apoptosis via Fas ligand (FasL, CD95L), or by release of proteolytic, or pore-forming enzymes.

4.2.6.1.4 Regulatory T cells

Treg are present in atherosclerotic lesions, but the levels of Treg are low in human plaques [22, 94]. Tregs have been shown to have a significant impact on atherosclerosis in murine models [23]. The signature cytokines of Tregs are TGFβ and IL-10. *Apoe*$^{-/-}$ mice expressing a dominant negative TGFβ receptor II in T cells, thereby disrupting TGFβ signaling, has increased atherosclerotic lesion size, increased IFNγ mRNA levels in the aorta, and increased macrophage and T-cell content in lesions [99]. Induction of Tregs decreases IFNγ levels and reduces lesion size [24]. This notion is supported by a study in *Apoe*$^{-/-}$ mice that showed that depletion of Treg by anti-CD25 antibodies increases atherosclerosis and increases macrophage and T cell content in lesions. The effect was abolished in mice lacking TGF-βII receptor in T cells, indicating that TGFβ is essential for Treg effects in atherosclerosis [100]. Bone marrow transfer of *IL-10*$^{+/+}$ cells to irradiated *Ldlr*$^{-/-}$ *x IL-10*$^{-/-}$ mice resulted in

increased lesion development [101], supporting an important role for IL-10 in the pathogenesis of atherosclerosis. In light of this, Treg remain an attractive target for immune manipulation in atherosclerosis treatment.

4.2.6.1.5 Th17 cells

Th17 cells are present in atherosclerosis and have been suggested to be both pro-atherogenic and atheroprotective [97]. Recent evidence suggest that TGFβ and IL-6, derived from the atherosclerotic plaques, can skew T-cell differentiation towards a Th17 cell phenotype in draining lymph nodes of the aorta, and that an increase in IL-17A can promote features associated with plaque stability [21].

4.2.6.2 B cells

Splenectomized *Apoe*$^{-/-}$ mice develop aggravated disease compared to controls, and transfer of B cells from *Apoe*$^{-/-}$ mice rescue and protect against disease [102]. In contrast, recent data indicate that depletion of B cells using anti-CD20 antibodies reduced lesions size in *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice [103]. The role of B cells in atherosclerosis is clearly complex, and one can speculate that the distribution of antibody production in terms of specificity and isotype might be important. Germline-coded antibodies have been suggested to be atheroprotective, but recent data suggest that these antibodies lack protective function, and the role of B cells in atherosclerosis is not completely clear [104]. In addition to antibody production, B cells can function as APC and secrete cytokines, functions that has been implicated in the pathogenesis of atherosclerosis [105].

4.2.6.3 Innate immunity in atherosclerosis

Granulocytes are important cells in the host defense against pathogens and the most abundant leukocyte in the circulation. Neutrophil granulocytes are present in the early stages of the development of the atherosclerotic lesion, and have also been observed in advanced stage lesions [106]. Neutrophils express TLR2, and *Tlr2* deficiency in *Ldlr*$^{-/-}$ mice decreases atherosclerosis [107]. TLR2 on ECs stimulated by neutrophils aggravates EC stress and may induce apoptosis, suggesting that neutrophils may provide a mechanism for plaque erosion [52]. Eosinophils are attracted to sites of inflammation by chemoattractants, such as CCL2, a chemoattractant expressed in atherosclerosis. Detecting eosinophils in atherosclerosis has been challenging, maybe due to short half-life. The contribution of eosinophils to atherosclerosis remains unclear [96].

Mast cells are present in atherosclerotic lesions from an early stage [44]. In healthy arteries, few mast cells are located in the adventitial layers. In atherosclerosis, mast cells accumulate in the adventitia and under the luminal endothelium, indicating that recruitment of mast cells occur from both the luminal side and from microvessels to the atheroma [108]. Through the release of proteases and cytokines such as IL-6 and IFNγ, mast cells are believed to contribute to the inflammatory milieu in the plaque [109, 110].

Macrophages derived from tissue-infiltrating monocytes are present at all stages of disease and constitute the major inflammatory cell type in the lesion [39, 44]. Monocytes patrol the arterial wall and migrate at sites as a response to chemokines, such as CCL-2 [111]. In atherosclerosis, mainly the $LY6^{hi}$ subset is recruited to the lesion, and monocytes then matures into macrophages or DCs. In addition, resident macrophages may also proliferate locally in the tissue [111]. Infiltration of monocytes is seen in early atherosclerosis and monocytes accumulate in the aortic root and thoracic aorta of *Apoe*$^{-/-}$ mice [96]. Macrophages in atherosclerosis are phagocytic cells that express TLRs [112], costimulatory molecules [6], and present antigens in a MHC class II restricted fashion to helper T cells. Activated macrophages secrete proteases and pro-inflammatory cytokines such as TNF and IL-1β, that contribute to increased inflammation and lesion development [96]. In atherosclerosis, both the classically activated macrophages induced by IFNγ, TNF, and lipopolysaccharide (LPS), and macrophages induced by other cytokines, such as IL-4 and IL-10 are abundant [113]. In addition, macrophages express costimulatory molecules such as CD80, CD86 [111] and CD137L [6]. When the macrophages die, they release lipids and tissue factor, both important for the pro-thrombotic features of the necrotic core [111].

Dendritic cells are professional APCs which are present in atherosclerotic lesions [114]. DCs communicate with the atherosclerotic lesion both through microvessels and by tissue migration in and around the atheroma. Upon antigen presentation to the adaptive immune cells, DC costimulate these cells, a process taking place in draining lymph nodes or in secondary lymphoid organs, and possibly also in tertiary lymphoid organs (TLO), potentially bringing T cell costimulation and adaptive immunity in very close proximity of atherosclerotic lesions [47] (Figure 2).

4.2.7 Current clinical practice

The anatomical characteristics of the vasculature are important determinants of the location of the atherosclerotic lesion. Typically, lesions develop at branches with turbulent flow, such as the carotid bifurcation. In contrast, sites with laminar flow are relatively resistant to early atherosclerosis development [51]. Although the flow itself is likely not responsible for lesion development, the hemodynamic activation of the endothelium is probably involved in the process leading to an atherosclerotic lesion [39, 51, 115, 116]. The location of the atherosclerotic lesion will affect the manifestation of the disease. For example, a plaque in the right coronary artery may affect sinus rhythm if blood flow to the sinoatrial node is disrupted, and a carotid plaque may embolize to the brain.

Atherothrombosis in a coronary artery may impair blood flow and delivery of oxygen and nutrients to the heart. The pulsatile contracting myocardium has a high demand of both oxygen and nutrients as reflected by the low oxygen saturation in sinus coronaries of approximately 30% [117]. The lack of oxygen and nutrients will first lead to impaired regional contractility of the heart, which may be manifested as heart failure. Another sometimes devastating complication is cardiac arrhythmias. Death of cardiomyocytes will lead to the release of markers of cellular damage, such as troponins.

Treatment of a myocardial infarction is based on three cornerstones; 1) platelet inhibition to prevent additional thrombus formation; 2) decrease of oxygen demand to prevent additional cellular death; and 3) revascularization to restore blood flow, oxygen delivery, and delivery of nutrients to the myocardium.

Rupture or erosion of a plaque in the carotid arteries may lead to a thromboembolic stroke, i.e. death of brain cells as a consequence of impaired blood flow in a vascular territory of the brain. The brain has a high oxygen and energy demand and is therefore very sensitive to any lack of blood supply.

Treatment of manifest stroke is principally based on restoring blood flow to the affected parts of the brain. This can be achieved either by dissolving, or by removal, of the thrombus. In addition, anti-coagulant drugs are an important treatment to counteract new thrombus formation.

Surgical removal of carotid atherosclerotic plaques is an established method for stroke prevention and was first done in the early 1950's [118]. Carotid endarterecomies are not used as an acute treatment, but rather a preventive measure for patients at risk for stroke, such as patients that suffered from a transitory ischemic attack (TIA). Interestingly, Rothwell *et al.* reported a decrease in stroke incidence. In combination with the reported changes in carotid plaque morphology by van Lammeren *et al.* we need to consider if this is change in course of disease and mechanism, i.e. an increased proportion of erosion rather than rupture of carotid plaques [65, 80]. It is tempting to draw the conclusion that the changes in CVD presentation are due to treatment with statins. However, life-style changes and healthcare improvement may also affect outcome, and any connection with change in disease needs to be proven in studies.

5 TUMOR NECROSIS FACTOR SUPERFAMILY

Receptors of the TNFRSF can be divided into two groups; 1) receptors with a death domain (DD, known as death receptors) that mediate apoptosis upon ligation, and 2) receptors interacting with TNF receptor associated factors (TRAF). TNFRSF are membrane bound, except two receptors always secreted as soluble proteins. Soluble forms of other TNFRSF, including CD137 (sCD137), are a result of cleavage or are generated by alternative splicing [119, 120].

Ligation of members of the TNFRSF results in either 1) apoptosis (e.g. ligation by TNF, CD95L), 2) proliferation (e.g. ligation by TNF, CD137L, OX40L), 3) differentiation (e.g. ligation by TNF, RANKL) or cell survival (e.g. ligation by RANKL) [120]. Ligation of different TNFRSF has receptor-type unique cellular effects despite common molecules in the signaling pathways [120]. Ligation of receptors, interacting with TRAFs, leads to the recruitment of TRAFs to the cytoplasmic tail of the receptor (Figure 3). Six TRAFs has been described in mammals, TRAF1-6 [120].

The molecule-specific responses of the different TNFSF/TNFRSF depend on a unique signature produced downstream of the ligation [2]. Furthermore, reverse signaling activating the ligand-expressing cell upon ligation of the receptor contributes to fine-tuning of responses [121]. This mechanism enables more plasticity of the immune response as it makes effector and stimulus codependent [121]. Reverse signaling can itself also change expression of costimulatory molecules. For example, ligation of OX40L on monocytes and DC by OX40 increases expression of other costimulatory molecules such as CD40, CD80, and CD86 [121, 122].

5.1 CD137 – REGULATION AND PATHOPHYSIOLOGY

5.1.1 CD137 – Regulation of expression

CD137 (alternative names 4-1BB, TNFRSF9) is a 28 kDa transmembrane receptor and the functional receptor is, like the ligand, trimerized on the cellular surface [123, 124]. Cells expressing CD137 are listed in table 2. CD137 is a CD28 independent costimulatory receptor of T cells [2]. CD137 is expressed on activated, but not on resting T cells [125]. Both $CD4^+$ and $CD8^+$ T cells express CD137, but stimulation of CD137 preferentially induces proliferation of $CD8^+$ T cells [126]. Furthermore, CD137 can also be expressed on Treg, and ligation of CD137 on Treg results in proliferation [9, 127]. The onset of CD137 expression in T cells upon activation is very fast. This expression can be prolonged with continued antigen exposure. CD137 upregulation is dependent on mitogen-activated protein kinases (MAPK) pathways, and T cell receptor ligation dependent CD137 regulation involves nuclear factor κ-light- chain enhancer of activated B cells (NFκB) and activating protein-1 (AP-1) (Figure 3) [128].

Table 2. Cells expressing CD137, CD137L, and OX40L.

	Cell type	Comment	Reference
CD137	Effector T cells	Expressed on activated T cells, mainly $CD8^+$	Pollok *et al.* 1993[125]; Kwon *et al.* 1987[129]
	Treg	Constitutively expressed in mice, inducable in humans	Goldstein *et al.* 2012[127]; Croft 2009[123]
	NK cells	Expressed upon activation	Melero *et al.* 1998[130]
	NKT cells	Expressed upon activation	Kim *et al.* 2008[131]
	EC	Expressed at sites of inflammation	Drenkhard *et al.* 2007[132]
	DC	Variable expression	Wilcox *et al.* 2002[133]
	Follicular DC		Pauly *et al.* 2002[134]
	Monocytes	Induces activation	Schwartz *et al.* 1995[135]; Kienzle *et al.* 2000[136]
	B cells	Promotes survival and proliferation	Schwartz *et al.* 1995[135]; Zhang *et al.* 2010[137]
	Mast cells	Augments secretion of IgE and cytokines	Nishimoto *et al.* 2005[138]
	Eosinophils		Heinish *et al.* 2001[139]
	Neutrophils	Expression in circulating neutrophils	Heinish *et al.* 2000[140]
	SMC	Variable expression	Broll *et al.* 2001[141]
CD137L	DC B cells Macrophages Monocytes	APC -Bidirectional activation	Shao, Schwartz 2010[142]; Langstein *et al.* 1998[143]
OX40L	DC B cells Macrophages	APC -Bidirectional activation	Croft 2009[123]; Lichtman 2012[1]; Gerdes, Zirlik 2011[144]
	SMC		Wang *et al.* 2005[145]
	EC		Nakano *et al.* 2010[12]; Wang *et al.* 2005[145]

Definitions are in the introductory list of abbreviations.

Vascular SMC in tumor tissue express CD137 as shown by immunohistochemistry in a study by Broll *et al.*, but the CD137 expression was variable between different tumor tissues [141]. Interestingly, CD137 deficient *Apoe*$^{-/-}$ mice show less apoptosis among vascular smooth muscle cells in the atherosclerotic lesions [146].

Ligation of CD40 by CD40L, molecules associated with increased inflammation in atherosclerosis [147], reduces the constitutive CD137 expression on DCs [148].

5.1.2 CD137 signal transduction

Ligation of CD137 leads to recruitment of TRAF1 and TRAF2, both required for maximal MAPK and NFκB activation in T cells (Figure 3) [2]. TRAFs are adaptor molecules needed to link the activated receptor to the intracellular signaling pathways [123]. TRAF2 is the major contributor to the classical (i.e. canonical) NFκB pathway responsible for fast response to stress stimuli [149]. Human CD137 can also recruit TRAF3 [150]. The alternative (i.e. non-canonical) NFκB activation pathway is normally slower in response (hours), due to the need of new protein synthesis. This latter pathway is usually suppressed by TRAF2. These mechanisms are hitherto most extensively studied for CD40-CD40L interactions. In this system, upon ligation of CD40, TRAF2 and 3 will be degraded, promoting an increase of NFκB inducing kinase (NIK) and, as a consequence, increased NFκB activity through the alternative pathway [8, 149]. The function of CD137 in the alternative pathway is unclear.

In summary, ligation of CD137 on T cells leads to TRAF-dependent NFκB activation and T cell expansion.

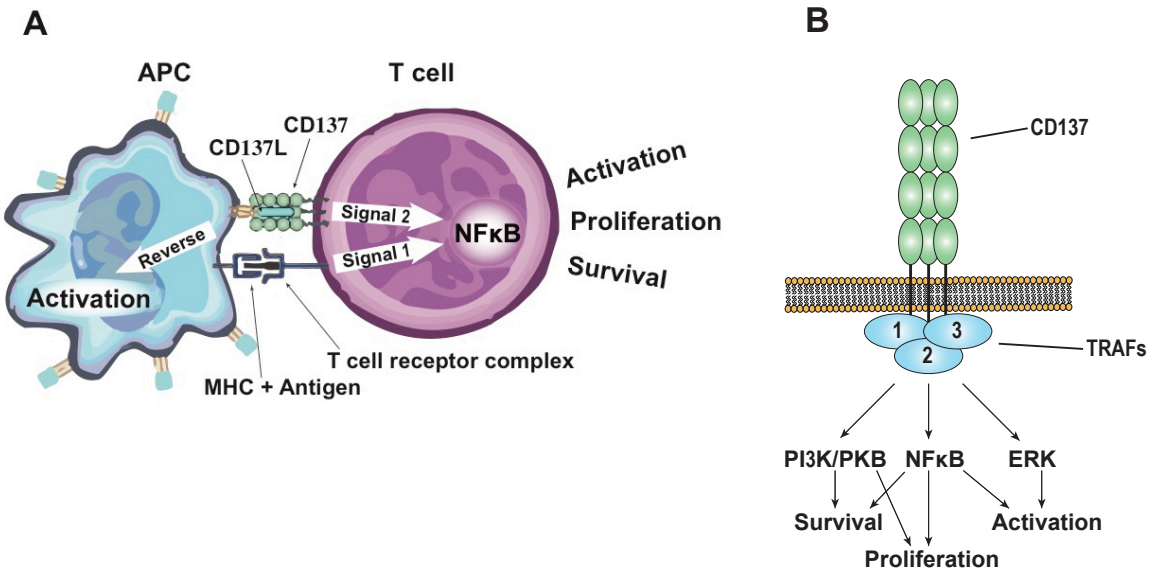

Figure 3. CD137/CD137L signaling. A) Reverse signaling of the ligand (CD137L) activates the APC at the same time as the ligand crosslinks the receptor (CD137). The T cell receptor complex provides signal 1 and the costimulatory receptor (CD137) provides signal 2, both needed for activation of the T cell. B) Signal transduction of CD137 in T cells.

5.1.3 Role of CD137 in experimental models of inflammatory diseases

There is an abundance of evidence that ligation of CD137 using agonistic antibodies contributes to amelioration of disease, in several models of autoimmune diseases.

Treatment with CD137-stimulating antibodies in models for Systemic Lupus Erythematosus (SLE) resulted in increased $CD8^+$ counts, ameliorated lymphadenopathy and splenomegaly, depletion of specific B cell clones, and markedly prolonged survival in mice due to reduced levels of auto-reactive antibodies [14, 151]. Furthermore, Vinay *et al.* showed that an SLE model deficient in CD137 has increased manifestations of SLE and increased mortality, suggesting a role for CD137 in experimental SLE development [152]. Treatment with a CD137 stimulating antibody in a model for experimental autoimmune encephalitis (EAE) resulted in reduced induction of EAE, and inhibition of disease relapse [13]. In the collagen induced arthritis (CIA) model, a mouse model for rheumatoid arthritis, administration of CD137 stimulating antibody blocked disease development, induced protective memory, and suppressed established disease [153, 154]. In addition, in a model for allergic asthma, administration of CD137-stimulating antibodies decreased allergen hyper-responsiveness and production of allergen-specific IgE, or even ameliorated disease [155, 156], effects that were abolished in *Cd137*$^{-/-}$ mice [157].

Hence, the totality of the available evidence implicates an important role for CD137 in a number of experimental models of autoimmune and inflammatory diseases. Considering the important role of inflammation in atherosclerosis pathophysiology, there is reason to examine the evidence on CD137 in vascular disease.

5.2 CD137 LIGAND – EXPRESSION AND FUNCTION

CD137 ligand (CD137L) was first described by Pollok *et al.* as a co-regulator of B cells [158]. CD137L is mainly expressed on the cell surface on activated APCs, and is a 27 kDa transmembrane protein with an intracellular N-terminus and an extracellular C-terminus [119, 142, 148]. In general CD137L expression is low. Under chronic inflammatory conditions, CD137L expression may be increased [159]. Interestingly, a feed-forward function has been suggested on both human and murine DC, where crosslinking of CD137L by CD137 increases enhances expression of CD137L itself, CD40, CD80 and CD86 [142].

CD137L activates both human and murine bone marrow derived macrophages and promotes expression of ICAM-1, M-CSF, and pro-inflammatory cytokines, such as TNF, IL-6 and IL-1β [142]. TLR4 activation induces CD137L expression, and endotoxin exposure of DCs promotes CD137L expression. Furthermore, a direct interaction between TLR4 and CD137L on the cell surface can promote TNF production in macrophages, indicating crosstalk between the innate and adaptive immunity [142, 160]. CD137L can be co-expressed with CD137 on activated murine and human T cells [161, 162], and exposure of human PBMC to either a CD137 decoy receptor, or antibodies targeting CD137L inhibits proliferation of T cells [142]. In addition, CD137L-stimulated DCs induce a stronger T cell response as supported by higher levels of IFNγ and increased proliferation [142].

Taken together, there is a strong foundation for CD137L expression in inflammatory milieus, such as the atherosclerotic lesion. Ligation of CD137 by CD137L activates both the APC and the receptor-expressing cell by bidirectional signaling.

5.2.1 Signal transduction and regulation of expression of CD137 ligand

A trimeric CD137L cross-links CD137 (receptor), thereby initiating the effects described with the receptor activation [123]. Besides activating CD137, CD137L also transmit signals into the cell on which it is expressed upon crosslinking the receptor (Figure 3). This feature is shared with several members of TNFSF and is referred to as reverse signaling or, together with the receptor signal, as bidirectional signaling [142]. Cross-linking of CD137L expressed on APC activates MAPK pathways, leading to NFκB activation and induction of pro-inflammatory cytokines, such as TNF, IL-1, IL-6, and IL-12 [123].

5.3 OX40 LIGAND – EXPRESSION AND SIGNALING

OX40 ligand (OX40L, alternative names: CD252, TNFSF4) is a 34 kDa glycosylated type 2 transmembrane protein expressed on activated APCs and endothelial cells. As with other TNFSF, a trimeric set of OX40L molecules bind to trimerized OX40 molecules [12, 123]. OX40 (CD134, TNFRSF4) is mainly expressed on activated T cells [2, 144]. In general, stimulation of the OX40L/OX40 pathway leads to clonal expansion and is important for long-lasting T cells responses [123]. OX40L/OX40 signaling increases survival of both effector and memory T cells, and to play a role in several inflammatory diseases, such as EAE [144]. Interestingly, OX40L/OX40 signaling inhibits development and function of regulatory T cells [10]. Like CD137L, OX40L ligation activates the cell by bidirectional signaling via the MAPK pathways. Ligation of OX40L leads to expression of cytokines, such as TNF, IL-1, IL-6, and IL-12. Furthermore, activation of OX40L in combination with TLR stimulation can lead to proliferation of DC and B cells [123, 163].

6 METHODOLOGICAL CONSIDERATIONS

The methods used in this thesis are in many respects standard methods in the field of medical research. However, all methods have their inherent strengths and weaknesses that need to be taken into consideration when assessing study design, results and conclusions.

6.1.1 Carotid lesions and control arteries

Human carotid lesions used in the studies included in this thesis are from the biobank of Karolinska endarterectomies (BiKE) cohort of peroperatively obtained carotid lesions [164, 165]. The plaques are from patients undergoing endarterectomy and plaques are handled and analyzed in a standardized manner to optimize for consistent quality of data. Patients in BiKE have lesions that are at an advanced stage of disease. Advanced lesions offer a unique opportunity to study late stage disease that is clinically relevant. When interpreting data from BiKE, disease stage needs to be taken into consideration since we are not comparing advanced lesions with lesions that would not be considered for surgery. The challenge of getting less advanced plaques is extremely hard, since benefits of surgery need to outweigh its risks and no one should be subjected to surgery without reasonable cause. The controls in the BiKE cohort are iliac arteries from organ donors and one aortic biopsy, all free from macroscopic atherosclerosis. An artery free from atherosclerosis normally contains very few infiltrating leukocytes and inflammatory activity is sparse. The renal arteries used as healthy arterial controls in this thesis were obtained from patients during nephrectomy due to kidney cancer. Macro and microscopic examination did not show any signs of vascular inflammation, but it is difficult to completely exclude that the pre-existing disease had some unknown effect on the artery.

Taken together, the included carotid lesions serves a good purpose for atherosclerosis studies and lots of knowledge has been derived from studying atherosclerotic lesions from carotid endarterectomies. Compared to studies on coronary atherosclerosis, which is mostly done on *post-mortem* specimens, peroperatively obtained carotid biopsies are a unique source of minimally degraded material. However, efforts should be made to improve human specimens for research, and there must be a continuous assessment of validity.

6.1.2 Human cohorts

HapMap is an international effort to describe the genetic variations in humans. Several populations were included in the project and different international research groups analyzed genotypes of the included subjects. In our study, we use the population of 30 trios of Utah residents of northern and western European ancestry (CEU) [166]. The publically available data was merged with data on gene expression in lymphoblastoid cell lines of the same population where CD137 mRNA levels were measured [167, 168]. At the time of the analysis, the combined data set offered an innovative and unique way to study the links between genotypes and CD137 mRNA expression. We performed our analysis using the available material at the time and identified one SNP of interest for CD137 expression,

rs2453021 in *CD137* [167]. This method does not describe protein expression, nor function or location, but offers a good opportunity for hypothesis generation for further investigation.

CASTRO is a population based survey of a nested case-control design and includes participants enrolled in two health surveys in northern Sweden. Blood samples and basic clinical data, such as blood pressure and comorbidity, were obtained at baseline. Cases used in our study were defined as ischemic first stroke before 75 years of age. DNA from 393 cases and 782 controls were available for analysis. 64 cases were excluded due to hemorrhagic stroke based on CT scan examinations, and 2 cases and 3 controls were excluded due to failed genotyping. CASTRO offers a unique opportunity to study genotype association with the incidence of stroke in a population based survey covering approximately 96% of strokes in the populations studied [169].

SAHLSIS is a Swedish study population of 600 patients with ischemic stroke and 600 matched controls. The subjects that suffered from ischemic stroke were categorized into subtypes according to etiology, i.e. large-vessel disease, small-vessel disease, cardioembolic stroke, cryptogenic stroke (undetermined cause despite extensive examination), other determined cause (cause that was known but did not fit into the defined categories), or undetermined cause (>1 cause or brief determination). SAHLSIS offers an opportunity to replicate the investigation in the CASTRO material in a similar population. Of note, in the SAHLSIS population investigated here, 98 of 600 individuals suffered from cardioembolic stroke [170, 171]. This multiplicity of causes might possibly reduce the clinical impact of a genotype since its effect on cardioembolic disease and vascular atherothrombosis may differ significantly. This should be taken into account when interpreting the findings of this study.

PROCARDIS is a European multicenter project that assembled a cohort that suffered from myocardial infarction or other acute coronary symptoms before 65 years of age [172]. The cohort has been genotyped by using either the Illumina Human 1M or 610K quad arrays and imputed to 1,000 genomes (CEU panel August 2009 release), and the subjects are phenotyped with clinical and biochemical data. In our analysis, imputed data was used for the study of CD137 since the investigated SNP (rs2453021) was not represented on the original array. Imputed data can be considered an accepted surrogate for testing the original SNP. In our study, there was no significant association between the imputed genotype data and coronary artery disease (CAD). Thus, the present results on imputed associations regarding the studied SNP (rs2453021) should be further investigated as more precise genetic data becomes available.

IMPROVE is a European multicenter study cohort of approximately 3400 individuals that had at least three risk factors for CVD but were free of CVD at enrollment [173]. The carotid arteries of the participants were examined using ultrasound and clinical laboratory data was collected. Participants were followed up during 36 months for evaluation of clinical cardiovascular events. The IMPROVE cohort represents a unique opportunity to study how genetic variations affect patients in relation to CVD. Ultrasound is a highly user-dependent technique where continuous assessment of findings is executed in real time by the examiner.

To minimize the inter-examiner-differences and inherent methodological variability, the IMPROVE cohort investigators use standardized examination protocols. Ultrasound examinations of the carotid arteries were performed using a frequency of 7-8 MHz and this frequency gives an axial resolution of 0.385mm and lateral resolution of 0.500mm. The study measured intima media thickness (IMT), which can be used as a marker of subclinical atherosclerosis. Importantly, IMT, i.e. a thickening of the innermost layer of the artery, is not necessarily correlated to an advanced atherosclerotic disease stage, but reveals a measurement of local atherosclerotic burden. In this way, measurement of IMT offers an opportunity to investigate development of atherosclerosis before onset of clinical CVD [174-176].

6.2 EXPERIMENTAL ATHEROSCLEROSIS

6.2.1 Apolipoprotein E deficient mice and the inducible plaque rupture model

To study mechanisms of atherosclerosis, a good model for disease is needed due to the obvious difficulties of setting up experiments in human subjects. Normally, mice do not develop atherosclerosis if not challenged with lipid rich diets for long periods of time. In this context, development of mouse models has been pivotal for atherosclerosis research [51]. Genetically modified mice with altered lipid metabolism are widely used in atherosclerotic research [39]. The mouse model in this thesis is the *Apoe*$^{-/-}$ mouse [177-179]. The lesions that develop in *Apoe*$^{-/-}$ mice share several features with human atherosclerotic lesions, for example the inflammatory phenotype and development of necrotic core in older mice [177, 180]. Rupture can occur in older mice, but rupture frequency is low, and the long time span makes the use of old mice difficult in investigations of plaque rupture mechanisms [146]. This has led to development of new models of plaque rupture based on the *Apoe*$^{-/-}$ mouse, where the carotid artery is partially ligated for atherosclerosis formation, and a conical cuff is placed around the ligated artery after 4 weeks to induce plaque rupture [181, 182]. This inducible plaque rupture model is one tool for the study of the rupture process. The exact mechanism inducing the rupture in this model is not fully understood, but changes in shear stress due to increased flow rate when narrowing the lumen has been suggested [181]. In humans, there is strong evidence pointing towards that increased inflammatory activity is important for plaque rupture [39, 45, 46]. In the inducible plaque rupture model, mice are at a relative young age at the time of euthanasia and the impact of inflammation on rupture is not clear [179, 182, 183]. In addition, recent evidence indicate that plaque erosion becomes increasingly important [43] and this model needs to be evaluated in this respect [70]. Although this model of plaque rupture does not perfectly replicate what likely is the most common pathogenesis of atherothrombosis in humans, it is useful to investigate particular aspects of vascular damage and atherothrombosis.

6.2.2 The 2A antibody

The CD137-stimulating antibody 2A used in the work of this thesis is a well-established antibody that has been used in other studies of inflammatory diseases [13]. When CD137 was targeted in models of inflammatory disease, several studies showed amelioration and sometimes reversal of established disease [13, 14, 154, 155]. The antibodies in the present studies were injected intraperitoneally and the antibodies are reasonably subsequently taken up into the circulation. The kinetics of the 2A antibody is not known, and optimal dose in the model has not been determined. The anatomical distribution of effects of the antibody is also not fully known and it is, therefore, currently unclear in which compartments the 2A effect is most pronounced and important. In our experiments, the antibody had the expected effects with increased proliferation and increased inflammation, i.e. replicating the immunological phenotype we set out to study.

6.3 ANALYSES

6.3.1 Semiquantitative real-time PCR (Taqman) and expression arrays

The measurement of mRNA levels in this thesis was performed using semiquantitative real time PCR (RT-PCR) (TaqMan) of target and reference transcripts, or gene arrays. Arrays offer a convenient platform for measurement of multiple molecule mRNA levels allowing for broad screening with a limited use of tissue. Since proteins are major performers of cellular functions, mRNA measurement is sometimes regarded as less informative. On the other hand, protein levels by themselves do not unequivocally predict function, efficacy, effect kinetics, and the influence of inhibitors and competition from other cellular processes. In light of this, measurement of mRNA levels does not provide a final answer about cellular functionality, but is a relevant source of information about changes in physiology and cellular function.

6.3.2 Single nucleotide polymorphism's – SNPs

Single nucleotide polymorphisms (SNP) are variations in a single nucleotide at a specific position in the genome. Variations defined as SNPs usually occur in ~1% or more of the population. Identification of SNPs in individuals can be performed by analyzing their DNA by PCR based technology or specialized DNA microarrays. An association between the presence of a SNP and a particular disease in a population provides statistical support for a connection between the genotype and disease development. Importantly, this relationship denotes an association between a SNP in a gene locus and disease, and does not prove an association with a gene or protein. To determine causality between the nucleotide variation and disease development, further investigation is needed. Therefore, statistical associations of SNPs with disease alone must be interpreted caution, but are valuable for hypothesis generation. Using these genetic association data together with experimental studies with a pre-formed hypothesis can be a fruitful approach to generation of new knowledge and advance our understanding of disease mechanisms.

The studies in this thesis analyzed genotypes and SNP using a hypothesis-driven approach, that is, a hypothesis was formulated based on the available data before the genomic data was queried and only one SNP was studied. Specifically, we and others first provided mechanistic data on TNFSF members in cardiovascular disease and the genetic regulation of CD137 in humans. Subsequently, hypotheses were formulated and tested in the available populations.

6.3.3 Immunohistochemistry

The basic principle used in immunohistochemistry is that primary antibodies bind to epitopes. Either a secondary antibody or another molecule that selectively detects the primary antibody subsequently detects antibody binding. Color is then used to analyze localization of the bound antibody. Immunohistochemistry can be used in combination with direct staining of tissue components (e.g. Sudan IV staining fat) for morphological visualization. These methods are used on a routine basis in clinical settings and has been a huge success in atherosclerotic research, maybe best exemplified by the discovery of inflammation in the atherosclerotic lesion [38]. The method has several advantages and weaknesses. The most striking advantage is the possibility to detect morphology and cellular location. Co-localization and physical proximity for cellular interaction can be revealed. The methods require highly specific antibodies. This is especially important when detecting molecules that have not been thoroughly investigated (methodologically) and when investigating molecules in complex tissues like atherosclerotic lesions where necrosis, fat and debris offer lots of unspecific false positive binding sites for the antibody. Results must therefore be evaluated with great care and sufficient control measures must be taken to evaluate the results. This includes relevant isotype controls, unstained sections and a thoughtful strategy. Perhaps most important of all, the results must be interpreted in the context and should be supported by other methods i.e. immunohistochemistry alone does often not offer sufficient evidence of expression, co-localization, and interactions.

6.4 CONCLUSION OF METHODS

To study a chronic life threatening disease, one would ideally study the disease in real time with a perfectly matched control and no confounding factors. One of the most obvious problems with this approach in atherosclerosis is that most of the study objects would outlive the researcher. Therefore, to advance knowledge and provide a basis for clinical protocols, we need to push our methodological knowledge forward and handle the inherited weaknesses of the methods available. The researcher has a huge advantage in the possibility to test a hypothesis, evaluate the result, and refine the experiment. This way, models and methods can improve continuously, and we can add new results to the bank of knowledge. Since atherosclerosis research ultimately strives to treat human disease, it is of great importance to evaluate all experimental data in the context of human using the available methods and samples.

The methods used in this thesis are by no means perfect, but they are well-established and recognized so that others can evaluate our results for transparence and sharing of knowledge.

7 EVIDENCE ON COSTIMULATION IN ATHEROSCLEROSIS FROM OUR EXPERIMENTAL STUDIES

The papers included in this thesis explore aspects of CD137 and OX40L in human and murine atherosclerosis. The focus of papers I, III and IV is CD137, and the focus of paper II is OX40L. Together these studies show that CD137 and OX40L are expressed in atherosclerosis and influence atherosclerosis pathophysiology.

7.1 CD137; PAPER I, III, IV

In an exploratory study with the aim to identify important molecules in the vascular inflammatory response, we incubated human arteries with endotoxin and measured effects on mRNA levels using Affymetrix gene array. This experiment identified CD137 as one of the most up-regulated genes (data not shown). The finding was surprising, because CD137 has mostly been considered a costimulatory molecule expressed on T cells, but since T cells are very rare in normal healthy arteries, we conjectured that the observed increased CD137 mRNA levels might have another cellular origin, and possibly play an unexpected role in the vascular inflammatory response and atherosclerosis. The findings caught our interest for many reasons. First, CD137, previously identified as a potent costimulatory molecule of T cells and also expressed by several other cells, had been shown to influence inflammatory diseases in mouse models of SLE and RA in a remarkable way with amelioration of disease and reports of regression of established disease [13, 154]. Furthermore, the non-resolving inflammation that is a hallmark of atherosclerosis has important features in common with other inflammatory diseases such as RA. The key role of T cells for orchestrating the pathogenic immune response in atherosclerosis further supported the interest in this CD28 independent T cell costimulator – especially now considering the reports on reduced CD28 expression in the elderly [184], a population in which cardiovascular disease is the major contributor to morbidity and mortality. Accordingly, we decided to investigate CD137 in human and murine atherosclerosis.

In human atherosclerotic arteries, CD137 mRNA levels were higher than in normal arteries, and incubation with LPS significantly increased CD137 mRNA levels. **Paper I** provided the first evidence of CD137 expression in human atherosclerosis. This study also discovered that CD137 expression was not limited to T cells in the atherosclerotic lesions, but it was also expressed on endothelial cells. There was no detectable expression of CD137 on any other cell type, which was somewhat surprising since CD137 is known to be expressed on several cell types (Table 2), of which the majority are present in atherosclerotic lesion. However, identification of the cell types was primarily done with immunofluorescence and the observed expression morphologically led us to investigate co-expression with markers of T cells and EC. Therefore, it is possible that other cell types expressing CD137 might be identified in atherosclerosis. In human atherosclerosis, CD137L staining co-localizes with CD68, indicating expression on macrophages [6, 185]. One of the most abundant cell types in

atherosclerosis is macrophages, suggesting an anatomical basis for CD137/CD137L interaction in lesions [6, 39, 42]. In patients with acute atherothrombotic stroke, Yu *et al.* observed increased levels of both CD137L expressed on circulating monocytes, and soluble CD137L (sCD137L) [186]. These results do not necessarily reflect the expression in atherosclerotic lesions, but higher levels of circulating sCD137L and membrane bound CD137L provides a foundation for increased CD137-CD137L crosslinking in these patients, although the conjectures about potential clinical effects on human atherosclerosis are yet distinctly speculative. Accordingly, in a series of subsequent experiments, we next asked whether activation of CD137 in experimental models had effects on vascular inflammation and atherosclerosis.

In vitro, CD137 mRNA levels were increased in response to inflammatory cytokines in cultured human umbilical vein endothelial cells (HUVEC) and aortic smooth muscle cells (AoSMC). We also observed that stimulation of CD137 using a trimerized recombinant CD137 ligand (trCD137L) promoted surface expression of vascular cellular adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1) in HUVEC. In cultured AoSMC, stimulation of CD137 with trCD137L inhibited AoSMC proliferation, an effect that was suppressed by the addition of the non-stimulating rCD137L monomer known to block ligand activation of CD137, thereby functioning as an antagonist. All this supported a role for CD137 in human atherosclerosis where the inflammatory milieu induces CD137 expression. In light of these observations, we proposed that the effects of CD137 activation might promote recruitment of inflammatory cells and increased plaque inflammation.

In patients with CVD, increased $CD4^{+}CD28^{null}$ T cells correlate with an increase in CRP and proinflammatory cytokines in peripheral blood [187-189]. This T cell subset does not appear in mouse models of atherosclerosis and is therefore not studied in these models. Interestingly, the specific subset of $CD4^{+}CD28^{null}$ T cells expresses high levels of CD137 and OX40 in patients with CVD, and these cells infiltrate atherosclerotic plaques. Furthermore, these CD137 expressing $CD4^{+}CD28^{null}$ T cells can change to a cytotoxic phenotype expressing perforin (CD161) [190, 191]. *In vitro*, the same subset of cells kill ECs, a process increased by CRP in a dose dependent manner [192], and the cytotoxic activity of $CD4^{+}CD28^{null}$ T cells in PBMC from patients with ACS can be activated or blocked by stimulation or blockade of CD137 respectively, supporting a possible role for CD137 in vascular damage [190, 191]. Interestingly, patients with ACS show increased plasma levels of soluble CD137 (sCD137), and levels correlate with increased troponin I, a marker for cardiac muscle injury [193], but the value of CD137 as a circulating biomarker is yet uncertain.

To further deepen our understanding of CD137 pathophysiology, we choose to study the effects of CD137 on atherosclerosis *in vivo*, and we turned to the hypercholesterolemic *Apoe*$^{-/-}$ mouse model of atherosclerosis. CD137 mRNA levels were 10-fold higher in aortas of *Apoe*$^{-/-}$ mice compared to wild type C57BL/6 mice used as a reference. Following injection of the CD137 agonistic antibody 2A in *Apoe*$^{-/-}$ mice, we observed an inflammatory phenotype as evidenced by increased mRNA levels of TNF, IFNγ, IL-1β, and ICAM-1 in the aorta. In

atherosclerotic lesions of the 2A treated mice, we observed an increased expression of CD3 and I-Ab. We also observed an infiltration of $CD8^+$ cells in the lesions. This is interesting since cytotoxic $CD8^+$ cells are rare in murine experimental atherosclerosis [4], but not uncommon in biopsies from human atherosclerotic lesions [38]. Moreover, Jeon *et al.* subsequently found that absence of CD137 in hypercholesterolemic mice resulted in decreased atherosclerosis and reduced inflammatory activity [185]. Furthermore, very old (>60 weeks of age) CD137 deficient hypercholesterolemic *Apoe*$^{-/-}$ (*Apoe*$^{-/-}$ *x Cd137*$^{-/-}$) mice had less atherosclerosis and reduced plaque rupture frequency compared to *Apoe*$^{-/-}$ mice of the same age [146], indicating a role for CD137 in plaque rupture. The mechanism for this inflammatory change in atherosclerosis has not been fully elucidated. In other models of inflammatory diseases, other investigators have observed that ligation of CD137 on Th17 T cells inhibits generation of more Th17 cells, favoring Treg. This is a suggested mechanism for the beneficial effects of CD137 activation in experimental some models of autoimmune diseases [20]. Interestingly, our observations suggest that other effects of CD137 activation may dominate in the pathogenesis of vascular inflammation, but the Th17/Treg ratio in our experimental was not investigated.

After establishing that CD137 and its natural ligand were present in atherosclerosis, and that stimulation of CD137 aggravated atherosclerosis in a mouse model, we became interested in studying CD137 in human atherosclerosis further. Therefore, we decided to investigate if genotype influences CD137 expression and, if so, if that identified genotype is associated with CVD. In **Paper III**, we discovered that the minor T allele of SNP rs2453021 was associated with decreased CD137 mRNA expression in immortalized lymphocytes in HapMap. The difference was significant in males, but only reached borderline significance in females. The selection of the rs2453021 SNP was done after analyzing the available SNPs and their effects on CD137 mRNA levels in the HapMap cohort in combination with an expression analysis of the same cohort [166, 168]. Based on our previous findings, and the results of the analysis, the SNP with a clear significant effect on CD137 expression was selected. We did not analyze the other available SNPs further in this study. The choice of studying CD137 genotypes was strictly hypothesis driven based on our previous results of CD137 in atherosclerosis. The findings of associations between the selected rs2453021 genotype should be considered in light of this basic premise.

We proceeded to investigate if presence of the minor allele of rs2453021 was associated with signs of cardiovascular disease. To this end, we queried a number of human cohorts. We turned to the IMPROVE cohort of more than 3400 patients with at least three risk factors for cardiovascular disease, but no clinical symptoms of CVD at enrollment. In the common carotid artery of participants in the IMPROVE cohort, presence of the minor allele of rs2453021was associated with an increased intima media thickness (IMT) at baseline and at 30 months follow-up. There was no significant association between rs2453021 and progression of IMT at the 30 months follow-up. When men and women were analyzed separately, the minor T-allele of rs2453021 was associated with higher mean IMT in men, but not in women, and with higher maximum IMT in women, but not in men. At the 36-

month follow-up of the IMPROVE cohort, prospective data on incident clinical events was analyzed. A total of 190 subjects suffered from one event or more during the 36 months. There were no significant associations with myocardial infarctions, stroke, or peripheral vascular disease when analyzed separately or all together. However, the minor T allele of rs2453021 was associated with increased risk for non-cardiac events. The choice of grouping the non-cardiac events was based on the number of clinical events during the follow-up and the need for statistical power. We observed non-significant trends for cerebrovascular and peripheral events and future follow-up studies in this cohort are warranted to clarify these trends. Of note, we observed that the minor T-allele of rs2453021 was associated with vascular events (all) in men, but not in women. This is interesting since other studies of TNFSF, e.g. OX40L, have shown differences between sexes in associations and CVD [145]. There was a significant difference in the CD137 mRNA levels between men and women. The underlying mechanism behind these differences is currently unknown. The minor T allele of rs2453021 was associated with the presence of plaque, defined as maximum IMT >1.5 mm, in the common carotid artery. This difference was abolished when men and women were analyzed separately, possibly due to loss of statistical power.

These results raised the question of potential associations between rs2453021 and clinical manifestations of CVD, and the PROCARDIS and WTCCC cohorts of more than 13000 cases and controls were analyzed for associations between rs2453021 minor T allele and CVD. There was no association between the minor T allele of rs2453021 and the incidence of myocardial infarction or CAD in the 13029 cases and controls of the PROCARDIS cohort together with WTCCC controls.

With the findings of CD137 in human atherosclerosis, the effects of CD137 stimulation in murine models of atherosclerosis, and the associations between the rs2453021 genotype and clinical disease in mind, the next step was to study the effects of CD137 on plaque rupture, considered the key event behind most clinical manifestations of CVD [43, 69]. To proceed, we turned to a murine model of inducible plaque rupture in **Paper IV**. We first confirmed that CD137 and CD137L mRNA levels are higher in atherosclerotic lesions compared to controls in both human and murine carotid atherosclerosis. In humans, we now compared the CD137 and CD137L mRNA levels between men and women, and between symptomatic and asymptomatic patients. There were no differences in CD137 or CD137L mRNA levels between symptomatic and asymptomatic patients, nor between men and women. In *Apoe*$^{-/-}$ mice, CD137 and CD137L mRNA levels were higher in ruptured carotid lesions than in non-ruptured. We also observed that mRNA levels of TNF, IFNγ, IL-1β, and MMP9 were elevated in ruptured murine carotid lesions compared to non-ruptured. Our previous *in vitro* data on human AoSMC and HUVEC showed that CD137 mRNA levels were higher in response to stimulation with inflammatory cytokines. If higher CD137 mRNA levels in the ruptured murine carotid plaques are induced by the inflammatory cytokines, or if the increased inflammatory milieu is caused by increased CD137 signaling remains unclear and needs to be addressed in a future study.

We also observed that *in vivo* treatment of *Apoe*$^{-/-}$ mice with the CD137 agonist 2A, increased the proportion of $CD8^+$ and a decreased proportion of $CD4^+$ splenocytes compared to IgG2a treated controls. We also observed that blood count of 2A treated mice were increased in granulocytes, proportion of monocytes, and frequency of mice with detectable eosinophils, compared to IgG2a treated controls. Furthermore, the proportion of circulating $CD8^+$ cells and the CD8/CD4 ratio were increased in 2A treated mice compared to IgG2a controls. These findings are in line with previously observed effects by 2A treatment on $CD8^+$ cells [6, 13, 154]. We also observed that activation of CD137 with the agonistic antibody 2A resulted in significantly higher mRNA levels of TNF, IFNγ, and IL-10 in the abdominal aorta. No significant effect on the levels of IL-1β, IL-5, IL-6, or TGFβ by the treatment was detected. These are expected effects of 2A treatment and the observations support that the integrity and administration of 2A was adequate.

To study the aortic atherosclerosis development in the experimental plaque rupture model, i.e. at an early disease time point in *Apoe*$^{-/-}$ mice, the thoracic aorta was pinned and stained. There was no significant difference in the atherosclerotic area between 2A and IgG2a treated mice as determined by oil-red-O staining. Importantly, the stained area in both groups was very low and ranged from 2-4%. It is very difficult to detect significant differences in disease development with this method in vessels that show this low fraction of stained area. Hence, we refrain from drawing conclusions about the 2A effect on atherosclerosis in this model at this time point.

To investigate the effects of CD137 stimulation on plaque rupture frequency, we used a mouse model where the right carotid artery was partially ligated and a subsequent placement over the ligated artery in *Apoe*$^{-/-}$ mice. Arteries were ligated at 12 weeks of age and the cuff was surgically placed at 16 weeks of age. Four days after cuff placement, mice were euthanized. At harvest, plaques were scored using visual inspection and, in select individuals, evaluated with immunohistochemical stainings. There was no significant difference in plaque rupture frequency between 2A and IgG2a treated mice in this study. This was an unexpected finding since previous results indicated increased atherosclerosis and inflammation following CD137 stimulation, and the 2A treatment in the present study showed the expected effects on the other analyzed tissues and blood. However, the model used in this study is based on an incomplete carotid ligation to induce an atherosclerotic lesion, and a subsequent placement of a conical cuff around the ligated artery to induce rupture. The exact mechanism responsible for the rupture in this model is not fully known, but increased shear stress has been suggested [181, 182]. The contribution of inflammation on plaque rupture in this model is not fully known, but it is conceivable that there may be a difference in the mechanistic details of the pathophysiology leading to plaque rupture in this model compared to the process in a plaque that has developed under a long period of time. This speculation requires further experimental studies for proper evaluation.

Interestingly, we observed a non-significant trend towards less rupture in the 2A treated group. Since the exact role and mechanism of CD137 in atherosclerosis is not known, we

speculate that this observation may be a result of the plaque dynamics and development during the very short time frame of atherosclerosis and rupture mechanisms in this model. In previous studies, we have demonstrated CD137 expression in human atherosclerosis and that a genotype was associated with increased risk for non-cardiac vascular events [6, 167]. The associated increase in non-cardiac vascular events in the previous study (Paper III) may not be due to plaque rupture, but plaque erosion. If and how erosion occurs in the used plaque rupture model in the present study (Paper IV) is not clear [51].

CD137 and CD137L mRNA levels are higher in atherosclerotic lesions compared to controls in both human and mice. In humans, there was no difference in terms of CD137 and CD137L mRNA levels between symptomatic and asymptomatic patients. In mice, there was a significant difference where both CD137 and CD137L mRNA levels were significantly higher in ruptured compared to non-ruptured carotid lesions. The observed difference between murine and human CD137 and CD137L expression patterns may suggest that there are differences in the mechanisms of disease. In humans, not all symptomatic patients suffer from rupture of the carotid plaques. Plaque erosion accounts for approximately one third of symptomatic carotid lesions [43, 45]. Atrial fibrillation is a frequent source of embolization to the brain and subsequent stroke and this needs to be considered when evaluating the study results. In the examined cohort, all patients that had undergone endarterectomy had advanced lesions that had developed over decades. In the examined murine plaques, CD137 mRNA levels were higher in ruptured vs. non-ruptured plaques. These plaques were thoroughly characterized with immunohistochemistry according to rupture/non-rupture, which might increase the precision of the results. The interpretation of the findings implicates a role for CD137 in atherosclerosis, and the exact function of CD137 in plaque rupture and plaque erosion needs further investigation.

7.2 OX40L; PAPER II

Several members of the TNFSF have been investigated in atherosclerosis [144]. OX40L (TNFSF4) is mainly expressed on APC and the natural ligand for OX40. OX40L/OX40 signaling is important for T cell clonal expansion and memory development [2] and, importantly, OX40L/OX40 signaling dampens Treg response [10], all effects that have been coupled to increased murine atherosclerosis. Interestingly, murine Treg constitutively express OX40 and CD137, but expression of these molecules are inducible in human Treg [123]. In experimental atherosclerosis, lack of OX40/OX40L signaling decreases atherosclerosis in *Apoe*$^{-/-}$ mice [12], and disruption of OX40/OX40L signaling with a neutralizing antibody decreases atherosclerosis in *Ldlr*$^{-/-}$ mice [194]. Mice with targeted mutations in *Ox40L* (*Tnfsf4*) have smaller lesions and mice with overexpression of *Ox40L* had larger lesions than controls. In humans, the minor allele of rs3850641 in *OX40L* was associated with increased risk for myocardial infarction in women [145] and this led us to investigate if the same genotype, i.e. rs3850641, was associated with increased risk for stroke. In **Paper II**, we investigate the expression of OX40L in human atherosclerosis, and the association between

the minor allele of rs3850641 SNP and risk for stroke in two independent Swedish patient cohorts.

OX40L was expressed in human atherosclerotic lesions. In carotid lesions of the BiKE cohort, OX40L mRNA levels were higher compared to healthy control arteries. Immunohistochemical stainings showed OX40L expression in plaques and immunofluorescent stainings demonstrated that OX40L expression co-localized with CD68, α-actin and von Willebrand Factor (vWF) in human plaques. The co-localization with vWF indicates expression on EC. Interestingly, the OX40L expression on EC was only noted in the intra-lesional part of the microvessels in the plaques. The importance of this observation is not clear, but the role of the microvessels has gained recognition and provides an anatomical foundation for communication between the deep layers of the atherosclerotic lesion and the blood stream. EC has been suggested to express OX40L constitutively [195]. In our study, we did not observe any expression of OX40L on EC facing the carotid lumen. Interestingly, *Apoe*$^{-/-}$ *x Ox40L*$^{-/-}$ mice develop less microvessels [12]. In *Apoe*$^{-/-}$ mice, OX40L expression has been shown on EC, SMC and macrophages in atherosclerotic lesions [145]. Since our previous findings of CD137 on EC in atherosclerosis revealed a heterogeneous expression, this may also be true for OX40L. Considering the constitutive expression of OX40L on EC previously reported by Mestas *et al.* [195], the lack of observed staining on EC might be a result of down-regulation. In cultured HUVEC, OX40L was induced by TNF stimulation, but not by LPS, suggesting a different response to stimuli compared to CD137. However, the lack of OX40L expression on EC facing the carotid lumen in this study is interesting since we show that OX40L mRNA levels are higher in EC after TNF stimulation, a cytokine that is expressed in atherosclerosis. The finding of OX40L expression on EC on atherosclerotic microvessels suggests that the milieu in the atherosclerotic plaque may vary at different locations within the plaque, and that this may influence OX40L expression. This finding also indicates that several mechanisms may be involved in controlling OX40L expression by EC in atherosclerosis.

Since the minor allele of rs3850641 in *OX40L (TNFSF4)* had been associated with increased risk for myocardial infarction in women, we proceeded to investigate the influence of rs3850641 and risk for stroke. The rs3850641 did not significantly influence OX40L mRNA levels in 130 patients in the BiKE cohort, neither when all patients were analyzed, nor when men and women were analyzed separately. We also investigated the associations and need for surgery. There was no association between the minor allele of rs3850641 and the need for carotid endarterectomy when comparing patients in BiKE with matched controls neither when analyzing all patients, nor when men and women were analyzed separately. There were no significant associations of rs3850641with levels of atherosclerosis-associated systemic risk factors white blood cell count, HbA1c, CRP, cholesterol or triglycerides in the BiKE cohort.

To investigate the associations between the minor allele of rs3850641 and the risk for stroke, we turned to clinical cohorts and replicated similar investigation in two independent Swedish

cohorts, SAHLSIS and CASTRO. There were no significant association of rs3850641 genotype and risk for stroke in any of the separate cohorts, nor when women and men were analyzed separately. To further increase sample size and statistical power, we pooled the samples from CASTRO and SAHLSIS and observed similar results. The pooled sample size used in this study was 2300 individuals, approximately two thirds of the sample size used in the study by Wang *et al.*, and may be insufficient for detection of and increased risk for stroke. However, our study was powered to detect an odds ratio of ≥1.25 with 80% power. Hence, based on these findings, a strong effect on the investigated genotype on the risk for stroke is unlikely.

Hemodynamic forces and natural curvatures of the vasculature results in different flow and shear stress on the endothelium [51]. These differences in part dictates the sites of atherosclerosis development together with recognized risk factors, such as serum lipids and oxidative stress [51]. Differences in shear stress affect the expression of at least 100 genes, of which approximately two-thirds are up regulated and one-third is down regulated [196]. Thus, due to different anatomical locations and vessel anatomy in the carotid vs. coronary circulation, it is possible that the observed association between rs3850641 and risk for myocardial infarction in women by Wang *et al.* was associated with changes in a vascular segment that show a different gene expression pattern and physiology than carotid plaques.

Taken together, our data show expression of OX40L in human atherosclerosis, but show no association between the presence of the minor allele of rs3850641 and increased risk for stroke. Although both myocardial infarction and stroke are commonly caused by atherosclerotic disease, the diseases at different locations may not be the same. We conclude that the effect of TNFSF members in atherosclerosis may differ with the anatomy and local physiology of the arterial tree. Further studies are warranted to elucidate the detailed mechanisms of TNFSF member influence on atherosclerotic disease.

In our genetic studies of OX40L and CD137, there was a discrepancy in the results on myocardial infarction and stroke. While the reason for this is yet not entirely clear, it interesting to note that carotid atherosclerosis differs from the coronary atherosclerosis. For example, severity of stenosis strongly predicts future events in carotid, but not in coronary atherosclerosis [197]. In carotid atherothrombosis, a thrombus is rarely present at the luminal plaque erosion or rupture, and if present, it is usually small. The bigger vessel size and higher volume flow rates may contribute to both the differences in plaque morphology and embolization downstream [74]. Although not the same, coronary and carotid atherosclerosis shares many features, such as basic structures and infiltration of macrophages and lymphocytes [43, 45, 65, 76].

7.3 A ROLE FOR CD137 AND OX40L IN CLINICAL MEDICINE?

Although there has been an improvement in care of patients with myocardial infarctions and stroke, this is still a main cause of death worldwide [198, 199], and there is a strong need for improved treatments. CD137 and OX40L play a role in experimental atherosclerosis and may

act as markers for severe carotid and coronary disease in humans [186, 193]. Pre-clinical studies on members of the TNFRSF/TNFSF have shown promising results, both in tumor biology and in CVD, suggesting that inhibition or stimulation of the TNFRSF/TNFSF could be a potential target for treatment of disease [147] (https://clinicaltrials.gov). Clinical trials, although promising, have revealed several problems that are not yet solved. For example, targeting TNFSF5 (CD154, CD40 ligand) was promising in murine models, but resulted in increased thrombosis due to expression on platelets [200]. In spite of this, the severity of the diseases, and potential benefits of manipulation of molecules of the TNFRSF/TNFSF, still drives development of new treatments targeting these molecules [4, 8, 201]. However, if the mechanistic details of CD137 and OX40 involvement in CVD are known, it is conceivable to use pharmacological means to manipulate their activity in efforts to prevent atherothrombotic events.

8 PERSPECTIVES

8.1 IMPORTANCE OF MOLECULAR BIOLOGY – THE NEED FOR NEW TOOLS

In general, we as scientists, are obliged to use a reductionist approach i.e. we choose to take out several factors influencing the investigated process to refine the effect of the particular mechanism or process we are studying. Nobel laureate Thomas Lindahl described how he could not accept the difference of the observed and expected results, a notion that led him to the discoveries of the DNA-repair mechanisms. One conclusion of this is that we constantly need to question all our results, methods and conceptions about biology, and develop new tools for investigation. Furthermore, as long as we get correct observations, the data we collect today will be valid over time, but the interpretation may change. For example, if we shook a rabbit 400 years ago, and measured heart rate, the result would be the same as if we shook a rabbit the same way today. The difference is our understanding of the process.

The complex human atherosclerotic disease takes decades to a lifetime for full development, and experimental models where a hypothesis can be tested are probably the best ways for studies of the disease. The challenge is then to interpret the obtained data, and translate the results into human disease or physiology, a process that hopefully leads to better understanding and treatment of human disease.

8.2 WE DO GET BETTER!

The clinical use of TNF-inhibitors has changed clinical picture of several inflammatory diseases, and revolutionized the clinical outcomes for many patients. Unfortunately, not all patients are relieved from their symptoms, and there are side effects with the treatments in clinical use. Therefore, we need to continue developing new concepts for treatment, for example by exploring nerve stimulation for immunological manipulation.

The quest for new treatments involves several methods, all with their inherent strengths and weaknesses, all used by the scientist to overcome an almost endless list of obstacles. Hopefully, the giant collective efforts put in to those methods by thousands of brilliant scientists will generate new approaches on how to diagnose and treat disease in new and better ways.

9 CONCLUSIONS

Conclusions from the studies in this thesis are that

- CD137 is expressed in advanced human atherosclerosis and activation of CD137 aggravates atherosclerosis in hypercholesterolemic mice

- a specific genotype influences CD137 expression and this variant is associated with the incidence of vascular disease

- activation of CD137 aggravates inflammation, but not plaque rupture frequency, in an experimental plaque rupture model

- OX40L is expressed on EC in microvessels in human atherosclerotic leisons, but the investigated OX40L genotype was not linked to an increased risk for stroke

10 ACKNOWLEDGEMENTS

Peder Olofsson, my main supervisor, for your fantastic enthusiasm and your ability to share it. Meeting you was a true life-changer, and teaming up with you in research has been an amazing experience. There are so many things I have you to thank for – I don't even know where to start. Your talent to adjust and your never ending positive energy is fantastic, and your sense for good quality in life and science is brilliant. The modified Feynman algorithm (the Olofsson algorithm!) together with the strategies for research are truly legendary. I have learned so much from your almost never-ending pinpointing of good and not-so-good parts in our projects, always positive and supportive, and always with a brilliant solution up your sleeve. This journey just keeps on getting better and I am so much looking forward to our next project! Last but not least, I thank you for being such a good friend – let's go scramble some eggs!

Göran Hansson for providing excellent research opportunities, and being so generous with your knowledge and wisdom. Your capabilities in getting a fast understanding of complex data, and your ability to grasp new concepts are second to none. It has been a true honor to get to know you and to have you as my supervisor, and I feel extremely privileged and proud to have worked in your lab.

Maria Klement, for supervision and generous support with my last project, and for your excellent abilities to spot details. Thanks for always being so generous with your time. My external mentor **Eddie Weitzberg** for your ingenious ways in viewing science, and for setting a new unofficial record in mentorssamtal. You combine research, clinical skills and judgment in a way I really admire. Our unit leader **Per Eriksson** for being generous with knowledge, support, and time. I always feel welcome when stepping into your office, and stepping out I feel that I am elucidated. Our vice chancellor **Anders Hamsten** for bringing in the momentum in my genetic study, and for your great ways of wording problems with elegance. **Magnus Bäck** for proving that you can be an excellent researcher and clinician at the same time. I really do appreciate your input and wise comments. **Allan Sirsjö** for contributions to the investigations of CD137 and for being so welcoming to new studies and projects. **Lieping Chen** for the donations of 2A antibodies and the 2A hybridoma -without these contributions, this thesis would have been very different.

David Konrad for being a boss that can communicate, for real. Your ways of relating to clinical duties and research are fantastic. Lets attend one more ultrasound course together! **Lars Irestedt** for believing in me. You have to be the most unselfish person ever. I do not know how to thank you enough – it is beyond what I can put into words here. My clinical supervisor **Johan Petersson** for your ingenious ways to humor, clinic, talks, and life-perspectives. I have learned so much from our conversations. **Kristina Hambraeus Jonzon** for being an extremely supportive ST-boss, and for handling feedback in the most professional way. **Lars Eriksson** for being a frontrunner in research at our clinic. Always inviting, always full of positive energy, and always open for discussions. To hang with you

on the ASA congress was a great experience, and your enthusiasm for science is really inspiring. **Eva Selldén** for your fantastic understanding and support when I really needed it. I am truly greatful.

Gabrielle Paulsson-Berne for great laughs and fantastic support. Thanks for sharing knowledge and research experience. It has been fun to share office! **Daniel Ketelhuth** for always having time to discuss protocols and scientific issues. Your skills in antibody production were invaluable. **Anton Gisterå** for being so supportive and helpful, both in the lab and in the animal house. And for dragging me to the gym! **Andrés Fernandés Laguna** for your fantastic attitude, your support with protocols, and for setting new standards for corridor sounds with your trumpet! **Marcello Petri, Konstantinos Polyzos, Roland Baumgartner** for help with dissections and more! **Karl Gertow** for great partnership in paper III. **Yuri Sheikine** for the most fascinating navigation skills in the northern suburbs of Stockholm, and for being such a good friend. **Jonas Persson** for god discussions and good clinical advice. **John Pirault** for your generous help in the lab, and your efforts to increase my knowledge of Languedoc-Roussillon. **Zhong-qun Yan** for inviting me to discussions from day one, i.e. 2005! **Sophie**, **Cheryl**, and **Yajuan** for fantastic skills, and for being such good collegues. **Norbert Gerdes, Roland Klingenberg, Anna Lundberg,** and **Hanna Agardh** for fantastic support and a sailing trip I will never forget. **Stephen Malin** for helping me with antibodies and good answers about how to write data. **Ediy Nagy, Olga Ovchinnikova, Daniel Johansson, Daniela and André Strodthof, Martin Halle, Tinna Christensdottir , Glykeria Karamidou, Reiner Mailer, Martin Berg, Maria Forteza, Miguel Carracedo, Silke Thul, Katrin Habir, Monica Centa, Albert Dahdah** – it is great to be in a great environment with fantastic people! **April Caravaca** for helping out with this part. **Fredrik Lennmyr** for the factor cover!

Annelie Olsson, **Ingrid Törnberg**, and **Linda Haglund** for all the excellent and skillful technical support in the lab. When you guys help me, I always know that things will turn out fine! **Inger Bodin** for fantastic support when I did my stainings.

Ann Hellström for fantastic support for me and the whole group. Nothing was ever a problem. My new roommate **Christina Hadders** for your positive attitude and for keeping our economics in good shape. **Linda Berglund** for managing our practicalities in a proper way.

Lars Maegdefessel, Hong Jin, Susanne Eken, Yuhuang Li, and the other members of Lars M's group for great collaborations and support. **Ulf Hedin** for you generosity with your time and for always being supportive. **Ljubica Perisic** for helping out with BiKE data, **Joy Roy**, and all other coworkers in the vascular surgery group for being helpful colleagues. **Maria Sabater Lleal**, for explaining genetics with such patience. **Rona Strawbridge** for even more discussions on genetics. All the helpful colleagues in the Per Eriksson group. **Cecilia Söderberg-Naucler** for your fantastic energy, **Afsar Rahbar** for help whenever I have asked for it. All friends and colleagues in the CMV group – it is a privilege to have good neighbours!

All co-authors on the papers – without you I would not be where I am today. Not even close. **Per-Gunnar Wiklund** and **Christina Jern** for your generous help with CASTRO and SAHLSIS.

Bo Ravn, my dear friend. The 6th line for on-call anesthesiologists is not replaceable. Your ways of describing our reality is second to none. **Andreas Wiklund**, for being my partner in crime in song writing. Working with you always inspires me to learn new things. **Andreas Hvarfner** for giving and taking feedback in a fantastic way. You share your expertise and invite to conversation at a fantastic level. To start a party with a precision slap bass duel with **Håkan Björne** will never ever be outdone by anyone. Period. You are brute positive force. **Bijan Darvish** for always being supportive. **Anders Oldner** for support and the discussions of clinical evidence, and together with **Magnus Falkenhav** making me realize what it takes to make things happen in the ICU. Your capabilities to foresee situations are magical. **Petter Westfeldt** for being such a great and supportive colleague. Lets flip the room! **Malin Jonsson Fagerlund** for good comments and discussions about pharmacology. **Peter Sackey** for making me feel inspired in the clinic and for having such a great mindset. **Kirsi Dolk** for making the schedule compatible with research. And for just knowing what is needed to make it to the thesis defence! I am truly greatful! **Eva Christensson** for your fantastic support and for stepping up and taking care of things. **Johan Nordström** for your positive energy and generosity with knowledge, and for always being so welcoming. **Peter Rudberg** for impersonating elegance. Can we share the Brett Sinclair-role at the clinic? **Tomas Öhman** for always bringing positive energy, a laugh, and music. **Stuart Duffin** –stepping out as a singer! Fantastic! **Jonas Blixt** for your great ways of reviewing knowledge and your inspiring pepper-passion about Chianti. The car is now repaired. **Andreas Gidlöf** for support at the clinic and the lab. **Kicki Hallin, Maggie Brohmée, Ann Norberg, Nadine Khammari, Ingeborg Gottlieb-Inacio** and the whole supportive team at ANOPIVA for your endless patience with me and practicalities. And to **all my dear co-workers at ANOPIVA** for making my job a fantastic experience every day!

Per Sandin, my dear friend since the beginning, for always being there to give me good advice, and for being a true friend for all these years. **Anna** and **Stefan Pettersson** for your fantastic support and generosity to my family and me. **Bengt** and **Ingbritt Bäcklund** for being the best mother in law and father in law that I could ever wish for. To my brother **Göran** and your family for support and stability. All **my old and new friends** making life fun and interesting!

To my **mom and dad** for being the best parents imaginable.

Karin and Emil for your endless support, love, and for all the hugs when I need it the most. For all the laughs and beautiful moments we constantly share. I love you.

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