Linköping University Medical Dissertations No. 1437

Pro- and anti-inflammatory actions in coronary artery disease

with focus on CD56⁺ T cells and Annexin A1

Ida Bergström

Linköping University
FACULTY OF HEALTH SCIENCES

Division of Cardiovascular medicine
Department of Medical and Health Sciences
Linköping University, Sweden

Linköping 2015

©Ida Bergström, 2015

Cover illustration by Anna F Söderström. Annexin A1 ribbon structure modified from original with kind permission from Dr. Anja Rosengarth, Department of Chemistry, Vanguard University, CA, USA. Illustrations on page 7, 17, and 34 by Dr. Emma Börgeson.

Published articles has been reprinted with the permission of the copyright holder.

Printed in Sweden by LiU-Tryck, Linköping, Sweden, 2015

ISBN 978-91-7519-150-8

ISSN 0345-0082

*To my family
because you are always there*

*“An experiment is a question which science poses to Nature,
and a measurement is the recording of Nature’s answer.”*

– Max Planck, 1858-1947

ABSTRACT

The atherosclerotic process is considered to be driven by an imbalance between pro- and anti-inflammatory actions. Still, the inflammatory state in patients with coronary artery disease (CAD) remains to be clarified. Annexin A1 (AnxA1) is a glucocorticoid-induced protein which may have a key role in the anti-inflammatory response as a mediator of glucocorticoid effects.

The general aim of this thesis was to deepen the knowledge of pro- and anti-inflammatory mechanisms in CAD via phenotypic assessments of immune cell subsets, in particular CD56⁺ T cells, and exploration of AnxA1. The long-term goal is to reveal basic mechanisms that will lead to the development of biomarkers, which may be used for individualized treatment and monitoring.

The AnxA1 protein was constitutively expressed in both neutrophils and peripheral blood mononuclear cells (PBMCs). However, it varied considerably across PBMC subsets, being most abundantly expressed in monocytes. The AnxA1 expression was also higher in CD56⁺ T cells than in CD56⁻ T cells.

The expression of total AnxA1 protein in neutrophils was higher in patients with stable angina (SA) compared with controls. However, this was not accompanied by altered neutrophil activation status. Instead, the neutrophils from patients exhibited an enhanced anti-inflammatory response to exogenous AnxA1, emphasizing the potential of AnxA1 as an inhibitor of neutrophil activity. Only patients with acute coronary syndrome (ACS) showed an increase in cell surface-associated AnxA1.

CAD patients, independent of clinical presentation, had increased proportions of CD56⁺ T cells compared with controls, a phenomenon likely to represent immunological aging. The CD56⁺ T cells were found to exhibit a distinct proinflammatory phenotype compared with CD56⁻ T cells. In all T cell subsets, the expression of cell surface-associated AnxA1 was significantly increased in ACS patients, while it tended to be increased in post-ACS patients. In addition, dexamethasone clearly inhibited activation of CD56⁺ T cells in *in vitro* assays, whereas AnxA1 did not. The findings highlight the need to clarify whether the role of AnxA1 is different in T cells than in innate immune cells.

In PBMCs, the mRNA levels of AnxA1 were increased in CAD patients, particularly in ACS patients. Correspondingly, the monocytes in ACS patients exhibited increased AnxA1 protein levels, both totally and on the cell surface.

However, only cell surface-associated AnxA1 in monocytes correlated with the glucocorticoid sensitivity of PBMCs *ex vivo*. We propose the expression of cell surface-associated AnxA1 to be a promising candidate marker of glucocorticoid sensitivity, which needs further investigations in larger cohorts and intervention trials. Furthermore, the fact that PBMCs in post-ACS patients exhibited proinflammatory activity but no increase in cell surface-associated AnxA1 allow us to speculate that the glucocorticoid action and/or availability might be insufficient in these patients.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Ateroskleros (eller s.k. åderförkalkning) kan drabba kranskärlen i hjärtat och yttra sig som antingen kärlkramp eller hjärtinfarkt. Ansamling av blodfetter i kärlväggen och aktivering av vita blodkroppar som skapar inflammation driver utvecklingen av sjukdomen framåt, medan inflammationshämmande substanser istället kan motverka sjukdomsprogressen. Kortisol är ett av kroppens främsta egna medel för att bromsa inflammation. Som svar på kortisol bildas annexin A1 (AnxA1), ett protein vars viktiga roll tros vara att förmedla kortisols inflammationshämmande effekter. AnxA1 i vita blodkroppar har studerats ganska lite vid olika sjukdomar och inte alls vid kranskärlssjukdom. Dess användbarhet i såväl diagnostik som behandling av inflammation diskuteras dock alltmer.

Syftet med denna avhandling var att mäta den inflammatoriska aktiviteten i olika vita blodkroppar hos patienter med kranskärlssjukdom och jämföra med friska individers. Vi mätte också förekomsten av AnxA1 i de vita blodkropparna och undersökte vidare hur kortisol och AnxA1 påverkade vita blodkroppars aktivitet.

När vi undersökte olika typer av vita blodkroppar såg vi klara tecken till ökad inflammatorisk aktivitet hos patienter med akut hjärtinfarkt men även hos de patienter som haft en hjärtinfarkt 6-12 månader tidigare. Innehållet av AnxA1 varierade betydligt mellan vita blodkroppar men ett generellt fynd var att vita blodkroppar hos patienter med akut hjärtinfarkt innehöll mycket mer AnxA1 än vita blodkroppar från friska individer. Hos patienter med tidigare genomgången hjärtinfarkt var dock skillnaden gentemot friska individer betydligt mindre.

Vi fann att kortisol effektivt kunde hämma den inflammatoriska aktiviteten i vita blodkroppar. AnxA1 hämmade också inflammation men detta var tydligt enbart i vissa typer av vita blodkroppar. I andra typer av vita blodkroppar (som dessutom förekom i ökat antal hos patienter) tycktes AnxA1 istället kunna motverka kortisols effekter. Detta är viktigt att studera ytterligare, särskilt med tanke på framtida användning av AnxA1 som ett eventuellt inflammationshämmande läkemedel.

Vi fann att mätning av AnxA1 i vissa typer av vita blodkroppar kunde ge information om hur effektiv den inflammationsdämpande effekten av kroppens

egen kortisolproduktion var. Våra fynd talade också för att den ökade inflammation som sågs i vita blodkroppar hos patienter med genomgången hjärtinfarkt skulle kunna höra samman med en otillräcklig kortisoleffekt. Dessa fynd behöver bekräftas i större studier men betyder att mätning av AnxA1 kan bli ett värdefullt verktyg i framtiden för att bedöma inflammationshämmande kapacitet hos patienter, något som nu saknas.

CONTENTS

ABSTRACT	V
POPULÄRVETENSKAPLIG SAMMANFATTNING	VII
LIST OF PAPERS	1
ABBREVIATIONS.....	3
INTRODUCTION.....	5
The immune system	5
Innate immunity.....	5
Adaptive immunity	7
Resolution of inflammation.....	8
Immune cells in blood	9
Neutrophils.....	10
Monocytes	11
NK cells	12
T cells	13
<i>CD4⁺ T cells</i>	13
<i>CD8⁺ T cells</i>	14
<i>CD56⁺ T cells</i>	14
Coronary artery disease	15
Atherosclerosis	17
Monocytes/macrophages in atherosclerosis	18
T cells in atherosclerosis	19
Other lymphocytes in atherosclerosis.....	20
Neutrophils in atherosclerosis	20
Anti-inflammation in atherosclerosis	21
Glucocorticoids.....	22
Glucocorticoids and coronary artery disease	23
Annexins	24
Annexin A1	25

<i>Annexin A1 in atherosclerosis</i>	<i>26</i>
AIMS	27
METHODOLOGICAL CONSIDERATIONS	29
Study populations.....	29
Paper I.....	30
Paper II	30
Paper III-IV	31
Laboratory methods.....	32
Summary of methods used in Paper I-IV	32
Flow cytometry	33
<i>General principles.....</i>	<i>33</i>
<i>Analysis of flow cytometry data</i>	<i>35</i>
Enzyme-linked immunosorbent assay (ELISA)	36
Luminex.....	37
TaqMan reverse transcription-quantitative polymerase chain reaction (RT-qPCR)	38
<i>In vitro</i> cell stimulation.....	38
Supplementary methods.....	39
<i>Cell surface-associated and total expression of AnxA1 in neutrophils</i>	<i>40</i>
Statistical methods.....	41
RESULTS AND DISCUSSION	43
AnxA1 expression in neutrophils	43
CD56⁺ T cells –functional phenotype and AnxA1 expression	47
AnxA1 expression in PBMCs in relation to glucocorticoid sensitivity ...	53
CONCLUDING REMARKS	57
ACKNOWLEDGEMENTS	59
REFERENCES	67

LIST OF PAPERS

This thesis is based on the following original publications, which will be referred to by their Roman numerals

- I. Eva Särndahl, **Ida Bergström**, Johnny Nijm, Tony Forslund, Mauro Perretti, Lena Jonasson. Enhanced neutrophil expression of annexin-1 in coronary artery disease.
Metabolism. 2010 Mar; 59(3):433-40.
- II. **Ida Bergström**, Karin Backteman, Anna K Lundberg, Jan Ernerudh, Lena Jonasson. Persistent accumulation of interferon- γ -producing CD8⁺CD56⁺ T cells in blood from patients with coronary artery disease.
Atherosclerosis. 2012 Oct; 224(2):515-20.
- III. **Ida Bergström**, Anna K Lundberg, Chris Reutelingsperger, Jan Ernerudh, Eva Särndahl, Lena Jonasson. Higher expression of annexin A1 in CD56⁺ than in CD56⁻ T cells. Potential implications for coronary artery disease.
Manuscript
- IV. **Ida Bergström**, Anna K Lundberg, Simon Jönsson, Jan Ernerudh, Eva Särndahl, Lena Jonasson. Annexin A1 expression in blood mononuclear cells – a potential marker of glucocorticoid activity in patients with coronary artery disease.
Manuscript

ABBREVIATIONS

ACS	Acute coronary syndrome
AnxA1	Annexin A1
APCs	Antigen-presenting cells
CAD	Coronary artery disease
CD	Cluster of differentiation
cDNA	complementary DNA
CMV	Cytomegalovirus
CRP	C-reactive protein
CVD	Cardiovascular disease
DAMPs	Damage-associated molecular patterns
DCs	Dendritic cells
ECG	Electrocardiogram
ELISA	Enzyme-linked immunosorbent assay
FSC	Forward-scattered light
FPRs	Formyl peptide receptors
FPR2/ALX	Formyl peptide receptor 2/receptor for lipoxin A ₄ and aspirin triggered lipoxins
GR	Glucocorticoid receptor
HDL	High density lipoprotein
HIV	Human immunodeficiency virus
HPA	Hypothalamic-pituitary-adrenal
HRP	Horseradish peroxidase
ICAMs	Intercellular adhesion molecules
IFN	Interferon
IL	Interleukin
KIRs	Killer inhibitory receptors
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
LTB ₄	Leukotriene B ₄
MFI	Median/Mean fluorescence intensity
MHC	Major histocompatibility complex
MI	Myocardial infarction
MMP	Matrix metalloproteinase
mRNA	messenger RNA
NETs	Neutrophil extracellular traps

NK	Natural killer
NLRs	Nucleotide-binding oligomerization domain-like receptors
oxLDL	Oxidized LDL
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PC	Phosphorylcholine
PE	Phytoerythrin
PMN	Polymorphonuclear leukocytes
PRRs	Pattern recognition receptors
RA	Rheumatoid arthritis
rAnxA1	Recombinant AnxA1
ROS	Reactive oxygen species
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
SA	Stable angina
SLE	Systemic lupus erythematosus
SMC	Smooth muscle cells
SSC	Side-scattered light
STEMI	ST-elevated myocardial infarction
TCR	T cell receptor
Th	T helper
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
T _{regs}	Regulatory T cells

INTRODUCTION

The immune system

The immune system is the body's defense against disease, and has evolved to protect us from infections and cancer in a versatile manner. Its crucial task is to distinguish between self and non-self molecules (or antigens) in order to orchestrate the up- and down-regulation of immune responses, and to eliminate disease while causing as little damage as possible to the host. The immune system comprises a variety of immune cells, as well as molecules such as cytokines, antibodies, and complement factors. It also consists of different types of organs, which are often divided into central (or primary) and peripheral (or secondary) lymphoid organs. The central lymphoid organs, *i.e.* the bone marrow and the thymus, are the sources of the immune cells. The secondary lymphoid organs are the lymph nodes, the lymphatic vessels, the lymph, the spleen, and the cutaneous and mucosal immune systems. In these organs, the interactions with antigens take place, adaptive immune responses are initiated, and recirculation of lymphocytes occurs. The functions of the immune system is organized into innate immunity, forming the primary protection against infections, and the adaptive immunity, which requires maturation into a more specialized defense (Abbas et al., 2014; Goldsby and Goldsby, 2003; Janeway, 2005).

Innate immunity

The innate immune system, also known as the natural or native immune system, is ready to start from birth and constitutes our first line of defense. It includes epithelial barriers (such as skin and mucosal membranes), soluble factors (complement system) and immune cells, granulocytes (*i.e.* neutrophils, basophils, and eosinophils), monocytes/macrophages, natural killer (NK) cells, mast cells, and dendritic cells (DCs) (Figure 1). When bacteria or other microorganisms pass the epithelial barriers, they are recognized by the innate

immune cells. The microbes carry pathogen-associated molecular patterns (PAMPs, *e.g.* lipopolysaccharide; LPS), which are recognized by pattern recognition receptors (PRRs, such as Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-like receptors (NLRs)) expressed by the immune cells. Activation of PAMPs through the PRRs leads to initiation of the immune response by the secretion of inflammatory mediators such as cytokines and chemokines, and by induction of different types of cell deaths (*e.g.* apoptosis, pyroptosis, NETosis). Another vital defense process performed by the phagocytic cells is phagocytosis of particles opsonized by complement and antibodies. Moreover, C-reactive protein (CRP) can opsonize microbes by itself, and activate the complement cascade. Also, the PAMPs can activate the complement cascade *per se*; resulting in lysis of the microbe. Secreted inflammatory mediators, like cytokines and chemokines, increase the permeability of the blood vessels and induce leukocyte adhesion and migration of first neutrophils and later monocytes to the site of infection. In the tissue, monocytes will differentiate into macrophages, and both neutrophils and macrophages will continue to do their duty, *i.e.* eliminating the pathogens (Abbas et al., 2014; Janeway, 2005).

The other types of innate immune cells also eliminate pathogens. NK cells kill cells infected with intracellular bacteria or viruses, as well as stressed cells displaying DNA damage or malignant transformations, by inducing apoptosis. The molecules released from stressed, damaged or necrotic cells are collectively called damage-associated molecular patterns (DAMPs), which are recognized by for example NLRs. The NK cells also secrete cytokines, thereby contributing to the regulation of the immune response. Mast cells can also be activated by PAMPs through TLRs, eliminating bacteria, and secrete cytokines or other inflammatory mediators. Together with eosinophils, they constitute a major defense against helminths, through degranulation and subsequent release of proteolytic enzymes and inflammatory mediators. DCs sense microbes and respond by secreting cytokines, but also by stimulating the adaptive immune responses and interacting with primarily T cells; thereby constituting the bridge between the innate and the adaptive immune responses. (Abbas et al., 2014).

Figure 1. An overview of the innate and the adaptive immune response. See text for explanations.

Adaptive immunity

The adaptive immune system, also referred to as the specific or acquired immune system, is more specialized and powerful than the innate system, but requires expansion and differentiation of lymphocytes and has therefore a slower on-set. It has often been divided into cell-mediated immunity, which involves T cells as a defense against intracellular pathogens, and humoral immunity, which includes B cells and the antibodies they produce as a defense against extracellular pathogens and pathogenic molecules (Figure 1). The adaptive immune response is initiated by antigen presentation by specialized cells called antigen-presenting cells (APCs), often, and in primary responses always, being DCs. The uptake of a pathogen activates the APCs and initiates their migration to the lymph nodes. In the lymph node the APCs display the

antigen to T cells, thereby activating the T cells and initiating clonal expansion and differentiation to effector cells and memory cells. Like NK cells, effector T cells with cytotoxic abilities induce apoptosis in cells infected with intracellular microbes. Effector T cells of T helper (Th) type, stimulate and enhance the abilities of phagocytes, secrete cytokines orchestrating the immune response, and also assist in B cell activation (Abbas et al., 2014; Janeway, 2005).

Most T cells are only able to recognize antigens of protein origin, whereas B cells can recognize antigens from many different types of molecules, like lipids, carbohydrates and nucleic acids. B cell activation is initiated by antigen binding, and processing followed by display for and recognition of Th cells, whereafter the B cell becomes fully activated. The Th cells not only mediate signals through cell contact with the B cells, but also secrete cytokines that are important for B cell activation. Upon activation, the B cells differentiate into antibody-producing plasma cells, or memory B cells. The antibodies mediate the extracellular defense by neutralizing and opsonizing pathogens, and by activating the complement system. As described above, the innate immunity is important in the early stages of the infection, whereas the adaptive immunity enhances the response if innate immunity fails. The adaptive response carries its own effector functions, but it mainly works by enhancing effector mechanisms in the innate immune system. Furthermore, the adaptive immune system has the ability of eliminating recurring or persistent infections through the generation of memory cells. When memory cells are activated by the pathogen that once induced their creation, they respond with the so-called secondary immune response, which is more rapidly and effectively eradicates the pathogen. As long as they do not encounter the pathogen that initiated their development, the memory cells are functionally inactive, and can survive for decades. Since new memory cells are generated for each new infection, there are very few in newborns but their numbers increase with age (Abbas et al., 2014; Janeway, 2005).

Resolution of inflammation

It is as important to achieve a successful resolution of the inflammation after pathogen destruction, as for the immune system to become activated when

pathogens are invading the host. The resolution is an active process, which neutralizes the tissue-injuring processes and agents, and restores homeostasis in the tissue. Failure of resolution may lead to chronic inflammatory conditions and autoimmunity. Cells as well as endogenous molecules mediate the resolution. The cellular processes include clearance of the pathogens that initiated the inflammation, clearance of pro-inflammatory debris, neutralization of pro-inflammatory chemokines and cytokines, phenotype switching of macrophages (from pro-inflammatory to pro-resolving), apoptosis of neutrophils and their subsequent phagocytosis by macrophages (*i.e.* efferocytosis), the exit of the macrophages from the site of inflammation, and recruitment of regulatory T cells (T_{regs}) in particular, but also NK cells and B cells. Natural antibodies, mainly produced by the B1-cell subset, are thought to participate in the elimination of apoptotic cells through their binding to “eat-me” signals, *e.g.* phosphorylcholine (PC)-containing lipids, thereby opsonizing the apoptotic cells. The endogenous molecules initiating the cellular processes of resolution include cytokines (like interleukin-10 (IL-10) and transforming growth factor- β (TGF- β)), pro-resolving proteins (like Annexin A1 (AnxA1)), and specialized pro-resolving mediators. The latter are small endogenously lipid-derived mediators, including lipoxins, resolvins, protectins and maresins (Klinker and Lundy, 2012; Ortega-Gomez et al., 2013; Panda and Ding, 2015; Serhan, 2014; Serhan et al., 2007; Serhan et al., 2010).

Immune cells in blood

All blood cells originate from pluripotent hematopoietic stem cells in the bone marrow, and can further differentiate depending on their myeloid or lymphoid origin. The myeloid progenitors are differentiated into erythrocytes, megakaryocytes (ancestors of platelets), granulocytes, monocytes, mast cells, and DCs. The lymphoid precursors give rise to T cells, B cells and NK cells. Blood cells are also commonly grouped into red blood cells (erythrocytes), white blood cells (all leukocytes), and platelets. The leukocytes are typically classified depending on their appearance in the light microscope into granulocytes, monocytes, and lymphocytes. The granulocytes include neutrophils, basophils and eosinophils, named after the staining properties of

different types of granules in the cytoplasm. The lymphocytes include all cells of lymphoid origin, *i.e.* T cells, B cells and NK cells (Janeway, 2005). The leukocyte subsets which are studied in this thesis are described more in detail, as follows.

Neutrophils

Neutrophils, also known as polymorphonuclear neutrophils/leukocytes (PMNs) or neutrophilic granulocytes, are the most abundant leukocyte subset in human blood representing around 60% of all leukocytes. The name arises from their granule content and multi-lobed nucleus. Neutrophil granules are traditionally divided into azurophilic granules (*e.g.* containing myeloperoxidase and elastase), specific granules (*e.g.* lactoferrin), and gelatinase granules (*e.g.* matrix metalloproteinase (MMP)-9). Neutrophils also contain secretory vesicles, which store different receptors such as cluster of differentiation (CD) 11b/CD18 (also known as β_2 -integrins) and formyl peptide receptors (FPRs). In blood, neutrophils are normally identified by their morphological characteristics. However, there are several markers used for identification, the most common being CD16 and CD66b in humans. Neutrophils are terminally differentiated in the bone marrow before being released into the blood. They are highly motile but relatively short-lived cells; remaining in the circulation up to approximately 10 h, and can survive 2-6 days in the tissue (Serhan et al., 2010; Wang and Arase, 2014).

As described above, neutrophils are crucial effector cells in the innate immune system. For neutrophils to migrate to the site of infection, an essential ability for the cell is to quickly shift from an inactive to an active and adherent state; a process mediated by cell adhesion molecules. When neutrophils get in contact with the activated endothelium, primary adhesion bonds between selectins and carbohydrates are formed. This primary adhesion, named tethering, make the neutrophils to slow down and to start rolling along the vessel wall. While rolling, the neutrophils are activated by chemoattractants (namely CXCL8, CXCL2, leukotriene B₄ (LTB₄) and fMLP), resulting in the up-regulation the adhesion molecules CD11b/CD18 on the cell surface that mediate a firmer, secondary adhesion by binding to intercellular adhesion molecules

(ICAMs) on nearby cells. The secondary adhesion causes arrest of neutrophils, and subsequent diapedesis, thereby enabling migration into the tissue (Wang and Arase, 2014; Witko-Sarsat et al., 2000).

At the site of inflammation, the neutrophils recognize PAMPs and DAMPs by PRRs and eliminate pathogens by induction of an inflammatory response, as previously described. The steps involved in phagocytosis are endocytosis of pathogen in a phagosome, fusion of phagosome with granules thereby forming a phagolysosome, and respiratory burst of reactive oxygen species (ROS). Neutrophils also possess another weapon for pathogen destruction, namely neutrophil extracellular traps (NETs). Furthermore, activated neutrophils influence inflammation by secreting various cytokines, chemokines and lipid mediators inducing either pro-inflammatory effects (CXCL8, IL-1 β and LTB₄) or anti-inflammatory effects (IL-1 receptor antagonist (RA), TGF β and lipoxins A₄ (LXA₄)). By this, neutrophils are able to support the inflammation as well as to promote its resolution. Moreover, neutrophils also contribute to the biosynthesis of resolvins and protectins. Clearance of neutrophils, i.e. neutrophil apoptosis and subsequent efferocytosis, is another crucial step for resolution; a process in which the neutrophils actively participate. Dying neutrophils send out “find me” signals that direct macrophages in their way, and also display “eat me” signals on their cell surface, AnxA1 being one of these signals (Jaillon et al., 2013; Ortega-Gomez et al., 2013; Serhan et al., 2010; Wang and Arase, 2014).

Monocytes

Monocytes represent around 10% of blood leukocytes in humans. After being released from the bone marrow, monocytes circulate in the blood for around 3 days. Traditionally, human monocytes have been identified based on their morphology together with the expression of surface marker CD14. In addition, CD16 has been used to further divide the monocytes into classical CD14⁺CD16⁻ and non-classical CD14^{low}CD16⁺ subsets. The classical CD14⁺CD16⁻ monocytes correspond to the LY6C^{high} monocytes in mice, while the non-classical CD14^{low}CD16⁺ monocytes match the LY6C^{low} murine monocytes (Ginhoux and Jung, 2014; Tacke and Randolph, 2006). Lately, also a third intermediate

monocyte subset has been described. The current consensus is to describe the monocyte phenotype for the classical monocytes as CD14⁺⁺CD16⁻, the intermediate as CD14⁺⁺CD16⁺, and the non-classical as CD14⁺CD16⁺⁺. Non-classical and intermediate monocytes can collectively be called CD16⁺ monocytes, if not separated. (Ziegler-Heitbrock et al., 2010). The vast majority of the circulating monocytes (approximately 85%) are classical, while around 10% are non-classical and 5% intermediate (Wong et al., 2012).

Functionally, the classical monocytes seem to be the main precursors to monocyte-derived macrophages, while non-classical monocytes are patrolling the luminal surface of the endothelium. Although tissue macrophages may be derived from monocytes, they may also originate from embryonic precursors present in tissues from birth. Furthermore, tissue macrophages are able to maintain their population by self-renewal, i.e. independently of monocytes. Some types of DCs are derived from circulating monocyte precursors. However, what kind of monocytes/monocyte precursors that give rise to different types of macrophages and DCs is still debated, and remains to be further clarified in humans (Ginhoux and Jung, 2014; Wong et al., 2012).

NK cells

Human NK cells are traditionally defined as expressing CD56 (or neural cell adhesion molecule) but lacking the T cell receptor CD3. Although classified as innate immune cells, they belong to the lymphocytes and account for about 10-15% of the lymphocyte population in blood. Compared to other lymphocytes, NK cells contain abundant granules (*e.g.* with perforin) in their cytoplasm. Upon detection of infected/damaged cells, the NK cells force the infected cell into apoptosis through release of granule contents and Fas-Fas ligand (L) interaction. NK cells express two types of receptors for controlling their cytotoxic activity, namely killer activating receptors such as NKG2D and CD16, and killer immunoglobulin-like receptors (KIRs). When NK cells encounter healthy cells, the KIRs will bind to and recognize MHC class I molecules thereby inhibiting NK cell activation. Upon activation, NK cells secrete proinflammatory cytokines, predominantly interferon- γ (IFN- γ) but also IL-12. Phenotypically, human NK cells are divided into two subsets depending on

their expression of CD56, CD56^{bright} and CD56^{dim}. The CD56^{bright} cells produce more cytokines, while CD56^{dim} are foremost cytotoxic cells. In blood, CD56^{dim} NK cells constitute > 90% of the NK cells (Abbas et al., 2014; Campbell and Hasegawa, 2013; Moretta et al., 2014; Serhan et al., 2010; Sun and Lanier, 2011).

T cells

T cells are originated from the bone marrow, but mature in the thymus, hence their name. All T cells express CD3, which forms a complex with the T cell receptor (TCR), a heterodimer of two chains, α and β . The induction of a T cell response is mediated through TCR recognition of antigens presented on MHC molecules. Through a selection process, it is ensured that T cells leaving the thymus do not recognize the host's own proteins, *i.e.* "self" antigens. Before the selection process, progenitor T cells are CD4⁺CD8⁻. The first selection steps check the composition of TCR, with successful rearrangements of first β , and then α chain. Thereafter, T cells become CD4⁺CD8⁺ and undergo positive and negative selection to check the functionality of the TCR. Only T cells that are able to moderately recognize MHC and antigens will survive, *i.e.* approximately 5% of all T cells. A too weak recognition of MHC and antigens is not associated with T cell survival since these cells are of no use for the immune system. On the other hand, a too strong recognition means risk for autoimmunity, and such cells are being forced into apoptosis. After the selection process, T cells become single-positive for either CD4 or CD8. If the TCR interacts with MHC class II, the T cell becomes CD4⁺, and if interacting with MHC class I, it becomes CD8⁺ (Abbas et al., 2014; Janeway, 2005).

CD4⁺ T cells

CD4 is a co-receptor to the TCR. T cells expressing CD4 are referred to as T helper cells, since they act mainly by "helping" other cells. The four most established CD4⁺-subpopulations are Th1, Th2, Th17 and T_{regs}. The Th1 cells produce proinflammatory type 1 cytokines, in particular IFN- γ , and are essential for cell mediated immune responses and important for elimination of intracellular microbes. Th2 cells producing cytokines, like IL-4, IL-5 and IL-13, are associated with the humoral immune response, and important for activation

of mast cells and eosinophils in the defense against helminths. Th17 cells are important effector cells against extracellular bacteria and fungi, and for neutrophil recruitment and activation through the secretion of IL17 A and F, but also IL-22. T_{regs} is a T cell subset that has gained much interest in the latest years, since T_{regs}, unlike the other CD4⁺ T cells, are immunosuppressive. They inhibit other immune cells either by cell to cell contact, or through the secretion of anti-inflammatory IL-10 and TGF- β . The correct phenotypic definition of T_{regs} is still a subject for debate, however they are traditionally characterized as CD4⁺CD25^{bright} and positive for the transcription factor Foxp3. (Abbas et al., 2014; Janeway, 2005; Serhan et al., 2010; Wan, 2010).

CD8⁺ T cells

The CD8⁺ T cells, commonly referred to as cytotoxic or cytolytic T cells, are essential for killing infected and tumor cells. Naïve CD8⁺ T cells are primed by APCs, and when antigen-specific CD8⁺ T cells recognize the appropriate peptide displayed on a MHC class I molecule with the TCR and co-receptor CD8, they are able to kill these cells. Activated CD8⁺ T cells secrete cytokines, mainly IFN- γ and TNF, in addition to killing of target cells. The CD8⁺ T cells mediate the cytolytically induced apoptosis using the same tools as NK cells, namely perforin, granzymes and Fas-FasL interaction. In the resolution phase, CD8⁺ T cells kill off other CD8⁺ T cells to restore homeostasis, a process called fratricide. CD8⁺ T cells have also been shown to produce IL-10 in order to prevent excessive tissue damage (Abbas et al., 2014; Janeway, 2005; Serhan et al., 2010; Zhang and Bevan, 2011).

CD56⁺ T cells

CD56, the NK cell marker, is also expressed by a subpopulation of T cells, the majority being CD8⁺. The CD56⁺ T cells are occasionally referred to as NKT-like cells but should not be mistaken for the true NKT cells, which express an invariant TCR and strongly respond to lipid antigens bound to CD1d (Peralbo et al., 2007). The expression of CD56 on T cells has been shown to closely correlate with cytolytic effector function, and higher production of IFN- γ (Ohkawa et al., 2001; Pittet et al., 2000). Moreover, CD56 can be acquired if

CD8⁺CD56⁻ T cells are exposed to IL-15. This *de novo* expression of CD56 occurs simultaneously as perforin/granzyme B acquisition and an increased expression of the anti-apoptotic Bcl-2 (Correia et al., 2011). CD56 expression on T cells is also associated with aging, since the numbers of CD8⁺CD56⁺ T cells are very low in infants but increase with age (Lemster et al., 2008; Pittet et al., 2000). Furthermore, the gain of CD56 has been shown to correlate with the loss of CD28, which is a hallmark of immunosenescence (Michel et al., 2007). While CD56⁺ T cells are potent producers of proinflammatory cytokines, such as IFN- γ and TNF, they seem to be poor producers of IL-10 (Katchar et al., 2005; Kelly-Rogers et al., 2006). Their functional role *in vivo* is still unclear but increased numbers of CD56⁺ T cells have been described in chronic inflammatory diseases, such as sarcoidosis, rheumatoid arthritis (RA), and Behcet's uveitis (Ahn et al., 2005; Katchar et al., 2005; Michel et al., 2007). However, there are also a few studies indicating that CD8⁺CD56⁺ T cells might have a suppressory and anti-inflammatory role (Davila et al., 2005; Shimamoto et al., 2007).

Coronary artery disease

According to the Global Burden of Disease Study 2013, cardiovascular disease (CVD) accounted for almost one third of all deaths in the world, with coronary artery disease (CAD, also named ischemic heart disease) being the main cause to premature mortality globally (Mortality and Causes of Death, 2014). In 2010, CAD was also found to be the main cause of disability-adjusted life years, fully explaining 5% of the total number of disability-adjusted life years worldwide (Murray et al., 2012). However, in high-income regions, reductions are seen in deaths from CVD (Mortality and Causes of Death, 2014), and the risk of dying from a myocardial infarction (MI) is the lowest in high-income countries (and highest in low-income countries) (Yusuf et al., 2014). According to the mortality database kept by the National Board of Health and Welfare in Sweden, the number of deaths due to acute MI was 6225 in 2013, a reduction by almost 40% since 2003. Taken together, the fact that having a MI in high-income countries is not as fatal as it once was, implicates that we are on the right track with prevention and treatment of CVD. However, much more work remains to be

done, since CAD is still the leading cause of mortality and morbidity in high-income countries and continues to increase in low-income countries.

Hypertension, hypercholesterolemia, male sex, obesity and smoking was identified as risk factors for CVD already in the Framingham studies (Dawber et al., 1959; Dawber et al., 1957; Kannel et al., 1961). The INTERHEART study further established these risk factors, and also identified diabetes, alcohol intake, psychosocial factors, low fruit and vegetable intake, and low physical activity to be associated with MI (Rosengren et al., 2004; Yusuf et al., 2004). High-risk patients exhibiting several risk factors can hereby be identified. However, it may still be difficult to estimate risk on an individual basis. Furthermore, many events occur in patients with a known history of CAD, despite revascularization and optimal medical treatment including the administration of platelet-inhibiting and cholesterol-lowering drugs.

CAD is normally divided into two different conditions; stable angina (SA), and acute coronary syndrome (ACS). The severity of symptoms in SA patients can be graded according to the Canadian Cardiovascular Society Classification (Table I) (Campeau, 1976). SA may remain in a stable (or even asymptomatic) condition, but can also turn into an unstable condition, or ACS. For some patients, ACS is the first clinical manifestation of CAD. ACS includes MI and unstable angina; the latter is characterized by sudden worsening of symptoms with severe, frequent, and prolonged angina also upon resting. The MI classification is based on typical changes in the electrocardiogram (ECG). An ST-elevated MI (STEMI), with ST-T segment depression and/or T-wave inversion, is the most severe MI condition, involving a total coronary occlusion, which needs immediate intervention, whereas non-STEMI is often caused by a partially but not completely blocked coronary artery. MI is separated from unstable angina by the elevation of troponins, reflecting damage to the myocardium (Thygesen et al., 2012).

The underlying cause of CAD is, in >90% of the cases, atherosclerosis (Qiao and Fishbein, 1991), which will now be further described.

Table I. Grading of angina pectoris using the Canadian Cardiovascular Society classification system

Grading	Description
Grade I	Asymptomatic or angina only during strenuous or prolonged physical activity
Grade II	Slight limitation, with angina only during vigorous physical activity
Grade III	Symptoms with everyday living activities, <i>i.e.</i> marked limitation
Grade IV	Inability to perform any activity without angina, or angina at rest, <i>i.e.</i> severe limitation

Atherosclerosis

Atherosclerosis was initially considered as a disease caused by passive cholesterol accumulation in the intima of the vessel wall. However, in the latest decades the role of inflammation has been established, and today atherosclerosis is considered not only as a lipid disorder, but also as a chronic inflammatory disease of large and medium-sized arteries. The accumulation of lipids and activation of innate as well as adaptive immune cells in the intima eventually leads to the formation of a plaque, characterized by a necrotic core that is surrounded by a fibrous cap of smooth muscle cells (SMC) and collagen (Figure 2). In the heart, plaque growth causes narrowing of the coronary artery lumen by > 70 % will induce myocardial ischemia, particularly in response to increased oxygen demand, and result in symptoms, such as effort-related angina pectoris, *i.e.* SA. However, in a worst case scenario, the weakening of the plaque fibrous cap leads to plaque destabilization and plaque rupture, with subsequent thrombus formation, thereby causing a life-threatening event of ACS (Hansson, 2005; Hansson and Libby, 2006; Libby, 2012).

Figure 2. A schematic view over factors and cells involved in atherosclerosis.

Monocytes/macrophages in atherosclerosis

A crucial initiating step of atherogenesis is the subendothelial retention of low-density lipoprotein (LDL) (Tabas et al., 2007). When LDL particles are retained by binding to proteoglycans in the extracellular matrix, they become susceptible for oxidation caused by ROS or enzymes. Several epitopes of the oxidized LDL (oxLDL) are to be considered as DAMPs, since they are recognized by PRRs, thus initiating an immune response (Miller et al., 2011).

OxLDL induces the expression of adhesion molecules, such as ICAM-1 and vascular cell-adhesion molecule (VCAM)-1 on the endothelium. Activated vascular cells also secrete chemokines, like CCL2 and CCL5, which initiate migration of monocytes into the vessel wall. Cytokines, (e.g. macrophage colony-stimulating factor) at the site of inflammation, induce monocyte differentiation into macrophages, and up-regulation of a specific type of PRRs collectively named scavenger receptors that will mediate the ingestion of oxLDL. The engulfment of lipids subsequently transforms the macrophages into so called foam cells. In the very beginning, accumulations of foam cells will form “fatty streaks” in the arterial wall. These fatty streaks can regress, but also progress to atherosclerotic plaques (Frostegard, 2013; Hansson and Hermansson, 2011; Weber and Noels, 2011; Virmani et al., 2000).

Lipid-laden macrophages display a defective efferocytosis, and are retained in the vessel wall due to reduced ability to egress, and hence inability to resolve inflammation. As the atherosclerotic process proceeds, accumulations of apoptotic and necrotic foam cells will result in the formation of a central necrotic core. Macrophages in the lesion also secrete pro-inflammatory cytokines, such as TNF, IL-1 β and IL-6. Moreover, they release other pro-inflammatory mediators, such as ROS and matrix-degrading proteases, which will further promote plaque growth and instability by affecting SMCs and surrounding matrix. (Hilgendorf et al., 2014; Moore et al., 2013).

In humans, a few studies have investigated the predictive value of circulating monocyte levels for cardiovascular events. In a population-based study, increased levels of classical CD14⁺⁺CD16⁻ monocytes independently predicted future cardiovascular events. Furthermore, a tendency ($P=0.051$) was seen also for increased numbers of intermediate CD14⁺⁺CD16⁺ monocytes in the cardiovascular event group (Berg et al., 2012). In a patient population referred to elective coronary angiography, only intermediate CD14⁺⁺CD16⁺ monocytes were found to independently predict cardiovascular events (Rogacev et al., 2012).

T cells in atherosclerosis

T cells can be detected at all stages of plaque formation. They are predominantly of effector/memory type, and recruited into the plaque in the same way as macrophages, *i.e.* through adhesion molecules and cytokines. Antigen-specific T cells are re-activated inside the plaque by cytokines, or by local antigen-presenting macrophages and DCs. Several antigens have been suggested to be important for development of atherosclerosis, such as heat-shock protein 60, and different epitopes of the oxLDL particle (Frostegard, 2013; Hansson and Hermansson, 2011; Weber and Noels, 2011).

A large number of experimental studies indicate that atherosclerosis is driven by a Th1-like type 1 response (Ait-Oufella et al., 2014). Pro-inflammatory cytokines such as IFN- γ and TNF have also been detected in human plaques (Frostegard et al., 1999; Methe et al., 2005), and according to histopathological studies, activated T cells are abundant in human lesions (Jonasson et al., 1986).

Moreover, raised numbers of IFN- γ -producing T cells have been found in peripheral blood in CAD patients, particularly in ACS patients; further indicating their potential role in plaque destabilization (Caligiuri et al., 1998; Methe et al., 2005; Neri Serneri et al., 1997). In human lesions, the CD4⁺ T cells predominate although CD8⁺ T cells are almost as abundant (Jonasson et al., 1986). There are implications for the involvement of CD8⁺ T cells both in early and late stages of atherosclerosis, and that the infiltration of CD8⁺ T cells increases with disease severity (Gewaltig et al., 2008; Kolbus et al., 2010). Interestingly, later studies have shown that human plaques contain more activated CD8⁺ T cells than activated CD4⁺ T cells (Grivel et al., 2011). However, compared with CD4⁺ T cells, the role of the CD8⁺ T cell compartment in atherosclerosis has been investigated in a lesser extent.

Other lymphocytes in atherosclerosis

B cells and NK cells can be detected in the atherosclerotic plaque, although in limited numbers (Packard et al., 2009). Functional studies have revealed that there are separate subsets of B cells, affecting atherosclerosis differently. The B1 cells can exert atheroprotective effects through their production and secretion of natural antibodies. In addition, B cells are able of producing the anti-atherogenic cytokine IL-10. On the other hand, other subsets of B cells secrete pathogenic antibodies, which may aggravate atherosclerosis. In humans, the different B cell subsets are not as clearly defined as in mice, due to lack of reliable surface markers (Perry et al., 2012; Tsiantoulas et al., 2014a; Tsiantoulas et al., 2014b).

The specific role of NK cells in atherosclerosis is still unknown. However, in patients with CAD, the numbers of NK cells in peripheral blood have been shown to be significantly lower than in healthy controls (Backteman et al., 2014; Hak et al., 2007; Jonasson et al., 2005).

Neutrophils in atherosclerosis

The role of neutrophils in atherosclerosis has been neglected for a long time, probably due to the low numbers detected in plaques. However, during the last

decade, this leukocyte subset has gained increased attention, and our knowledge in this area has expanded. Several studies have shown correlations between neutrophil counts and the development of ACS (Buffon et al., 2002; Friedman et al., 1974; Naruko et al., 2002; Ott et al., 1996). Neutrophil counts are also positively correlated with coronary atherosclerosis (Kostis et al., 1984), and numbers of neutrophil-platelet aggregates in peripheral blood (an indicator of neutrophil activation) have been shown to be increased in stable CAD patients (Nijm et al., 2005). In human atherosclerotic plaques, neutrophils are found in rupture-prone areas (Ionita et al., 2010). Inside the plaque, neutrophils are likely to contribute to plaque instability in multiple ways. By activation and release of ROS, they contribute to the oxidation of LDL, and through release of NETs they activate DCs. Moreover, neutrophils release several matrix degrading components, such as MMP-9 and elastase (Doring et al., 2014; Hartwig et al., 2014; Soehnlein, 2012; Weber and Noels, 2011).

Anti-inflammation in atherosclerosis

In experimental models, T_{regs} have been shown to be atheroprotective (Ait-Oufella et al., 2006; Mor et al., 2007). In patients with ACS, decreased numbers of T_{regs} in blood have been reported (Cheng et al., 2008; Han et al., 2007; Mor et al., 2006). Moreover, in a population-based study, low numbers of T_{regs} were associated with increased risk for developing acute MI, independent of other cardiovascular risk factors (Wigren et al., 2012). The T_{regs} are large producers of the anti-inflammatory cytokine IL-10, which in animal models has well-documented atheroprotective effects (Ait-Oufella et al., 2011). However, high levels of IL-10 in the circulation are reported in ACS patients, and somewhat paradoxically, IL-10 levels have been shown to predict risk for cardiovascular events (Malarstig et al., 2008; Patel et al., 2009). IL-10 is produced concomitantly with proinflammatory cytokines, and its presence in plasma does not seem to be a reliable marker of anti-inflammatory actions.

As previously mentioned, natural antibodies produced by the B1 cells have anti-inflammatory effects in atherosclerosis. High levels of antibodies against PC, which is present on oxLDL, have been associated with decreased for cardiovascular events (Caidahl et al., 2013; Gigante et al., 2014).

Glucocorticoids

In humans, cortisol is the predominant glucocorticoid hormone, whereas corticosterone is the predominant glucocorticoid in rodents. The release of cortisol is regulated by the hypothalamic-pituitary-adrenal (HPA)-axis. Briefly, corticotropin-releasing hormone (CRH), produced by and released from hypothalamus, mediates the secretion of adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland. ACTH subsequently stimulates the adrenal glands, inducing the synthesis and release of cortisol from the adrenal medulla into the circulation. The homeostasis of cortisol levels in blood is regulated by the negative feedback loop, where cortisol suppresses both CRH secretion from the hypothalamus as well as ACTH secretion from the pituitary gland. Locally, the cortisol levels are also regulated by the 11β -hydroxysteroid dehydrogenase enzymes, which converts cortisol to the inactive metabolite cortisone, and conversely cortisone to cortisol (Buckingham, 2006; Rang et al., 2007).

In healthy humans, the cortisol levels follow a robust diurnal rhythm, peaking in the morning after awakening. Cortisol levels gradually decrease during the day, reaching the lowest levels during the night around 2 - 3 AM, although increased levels are seen in response to meals, exercise, or threats (Buckingham, 2006).

Salivary cortisol shows the same diurnal pattern as cortisol in blood, and increase of cortisol in blood is rapidly reflected in saliva. Measuring salivary cortisol is a better way to determine diurnal variations in cortisol levels than blood cortisol analyses, since blood sampling is dependent on venous cannulation. However, in order to receive reliable data of diurnal variation, measurements over a period of 2-6 consecutive days is preferable (Aardal and Holm, 1995; Hellhammer et al., 2007; Kirschbaum and Hellhammer, 1999; Pruessner et al., 1997).

Glucocorticoids regulate numerous processes affecting metabolism, musculoskeletal homeostasis, cardiovascular function, cognition, reproduction, and of importance for this thesis –the immune system. Cortisol is the main endogenous anti-inflammatory mediator in the body, dampening both systemic and local inflammatory processes, thereby facilitating proper resolution of the inflammation. Due to their anti-inflammatory properties, glucocorticoids have

been extensively used as treatment for inflammatory conditions, and at pharmacological doses, they act immunosuppressively on basically all cells and mediators involved in immune and inflammatory responses (Buckingham, 2006; Perretti and D'Acquisto, 2009; Rang et al., 2007). Physiological concentrations of glucocorticoids rather show immunomodulatory effects, *e.g.* suppression of the type 1 immune responses by reducing T cell responsiveness to IL-12, inhibiting production of IFN- γ , while inducing IL-10 production (Fahey et al., 2006; Franchimont et al., 2000; Wu et al., 1998).

Glucocorticoids mediate their genomic actions through intracellular glucocorticoid receptors (GRs). After diffusion across the cell membrane, glucocorticoids bind to and activate the GR in the cytoplasm. After conformational change, the glucocorticoid-GR complex translocates into the nucleus where it binds to glucocorticoid response elements on glucocorticoid-responsive genes. Subsequently, multiple inflammatory genes are inhibited through the suppression of transcription factors, such as nuclear factor κ B and activator protein 1. On the other hand, the transcription of genes coding for anti-inflammatory proteins, such as annexin A1 and IL-10, is up-regulated. The GRs are derived from one single gene that by alternative splicing gives rise to multiple GR proteins: GR- α , GR- β , GR- γ , GR-A, and GR-P. In humans, GR- α and GR- β are the most widely studied isoforms. GR- α binds to and mediates the actions of glucocorticoids, while GR- β is a dominant negative inhibitor of glucocorticoid effects; antagonizing the binding of activated GR- α to DNA, thereby forming inactive GR- α /GR- β -complexes. (Kadmiel and Cidlowski, 2013; Oakley and Cidlowski, 2013).

Glucocorticoids can also act rapidly (*i.e.* within minutes) through non-genomic mechanisms, involving activation of mitogen-activated protein kinase pathways by cytoplasmic or membrane bound GRs (Ayroldi et al., 2012; Busillo and Cidlowski, 2013; Samarasinghe et al., 2012).

Glucocorticoids and coronary artery disease

Perturbation of the HPA axis function and impairment in glucocorticoid responsiveness, have been associated with various chronic inflammatory conditions (Silverman and Sternberg, 2012). Moreover, excessive levels of

glucocorticoids, either endogenous or exogenous (as in Cushing's syndrome), have been associated with hypertension, hyperlipidemia, hyperglycemia and abdominal obesity, and also with increased risk for cardiovascular events (Pimenta et al., 2012; Whitworth et al., 2005). However, it is not clear whether the increased cardiovascular risk is due to direct or indirect effects of glucocorticoids. Total cortisol exposure has been associated with subclinical atherosclerosis in the carotid arteries (Dekker et al., 2008). In a population-based study using repeated measurements of salivary cortisol during one day, a flattened diurnal cortisol slope was associated with coronary calcification (Matthews et al., 2006). Studies on cortisol levels in CAD patients are sparse and somewhat contradictory. In one study, higher serum cortisol in the morning was related to coronary stenosis in women with ACS (Koertge et al., 2002). In another study, CAD patients exhibited a higher total output of cortisol, and a flatter diurnal cortisol slope due to higher cortisol levels in the evening, compared with healthy controls (Nijm et al., 2007). It has been suggested that increased inflammatory activity in CAD patients may be a result of a dysfunctional HPA axis, and failure to resolve inflammation (Fantidis et al., 2002; Nijm et al., 2007).

Annexins

The annexins are a family of Ca^{2+} -dependent phospholipid-binding proteins consisting of 12 members in humans: AnxA1-A11 and A13. They are generally found in the cytosol, but can also be translocated to the cell surface. The annexins consist of a highly conserved C-terminal core domain of four similar repeats (six in AnxA6) of approximately 70 amino acid length forming a slightly curved disc of α -helices. The Ca^{2+} - and membrane binding sites are found on the convex side of the disc. The N-terminal is variable in sequence and length, more flexible, and located on the concave side of the disc. Although the N-terminal domain is a separate unit, it can be folded into the core domain, and it has been suggested to constitute the inactive form of the protein. Upon Ca^{2+} -binding, the N-terminal domain can be expelled from the core, and available for interaction. Despite their structural similarities, the annexins exert different functions

involving apoptosis, vesicle trafficking, cell division and growth (Gerke and Moss, 2002; Moss and Morgan, 2004; Rosengarth and Luecke, 2003).

Although none of the annexins have been proven solely responsible for causing disease, changes in annexin levels or localization have been linked to several diseases, such as cancers, autoimmune diseases and cardiovascular disease (Hayes and Moss, 2004; Wang et al., 2014). AnxA5 has recently been shown to exert anti-atherosclerotic effects, decreasing plaque inflammation, inhibiting pro-inflammatory immune cells and cytokines, and inducing anti-inflammatory cytokines (Burgmaier et al., 2014; Liu et al., 2015). AnxA1 and its potential role in cardiovascular disease will be discussed below.

Annexin A1

The most widely studied annexin in the context of inflammation is AnxA1, previously known as lipocortin-1, lipomodulin, renocortin or macrocortin. AnxA1 is an important player in the resolution of inflammation, and also a mediator of the anti-inflammatory effects of glucocorticoids. Normally, AnxA1 resides in the cytosol, but upon cell activation it is translocated to the cell surface, where it exerts its autocrine and paracrine actions. The effects of AnxA1 are mediated by the binding to formyl peptide receptor 2/receptor for lipoxin A₄ and aspirin triggered lipoxins (FPR2/ALX; previously called FPRL-1), a seven-transmembrane spanning G-protein-coupled receptor. Glucocorticoids induce expression of AnxA1, and also contribute to the translocation of the protein from the cytosol to the cell surface (D'Acunto et al., 2014; Perretti and D'Acquisto, 2009; Yazid et al., 2012). In healthy humans, AnxA1 is abundantly expressed in both neutrophils and monocytes, but much less in lymphocytes (Morand et al., 1995; Spurr et al., 2011). However, the AnxA1 expression seems to vary across the lymphocyte population, and according to an earlier study, CD56⁺ cells expressed the highest levels of AnxA1 among the lymphocyte subsets (Morand et al., 1995).

So far, most studies have investigated the role of AnxA1 in innate immune cells, more specifically in neutrophils, consistently demonstrating anti-inflammatory actions of AnxA1. AnxA1 has been shown to reduce both acute and chronic inflammation by inhibiting neutrophil adherence and migration,

and inducing neutrophil apoptosis, and also to function as an “eat me” signal promoting proper efferocytosis (D'Acquisto et al., 2008b; Perretti and Flower, 2004; Perretti and Solito, 2004). Furthermore, AnxA1 induces L-selectin shedding from human monocytes (Strausbaugh and Rosen, 2001), and inhibits the expression of IL-6 and TNF, while it stimulates the release of IL-10 from macrophages (Ferlazzo et al., 2003; Yang et al., 2009). Neutrophil expression of AnxA1 *in vivo* has also been shown to correlate to serum cortisol levels, which raised the hypothesis that AnxA1 expression in leukocytes could be used as a marker for tissue sensitivity to endogenous glucocorticoids (Mulla et al., 2005).

While the effects of AnxA1 in innate immunity are well documented, the role of AnxA1 in adaptive immunity is less studied. Moreover, the studies of AnxA1 in T cells have provided contradictory results. Some studies have shown an inhibitory role of AnxA1 regarding T cell-driven inflammatory responses (Gold et al., 1996; Kamal et al., 2001; Yang et al., 2013), while others report the opposite, *i.e.* AnxA1 as a promoter of T cell activation and proliferation (D'Acquisto et al., 2007a; D'Acquisto et al., 2007b; Paschalidis et al., 2009). As reported by D'Acquisto and coworkers, both the mRNA and protein AnxA1 expression in CD4⁺ T cells is higher in patients with RA compared with healthy controls (D'Acquisto et al., 2007a), and moreover, glucocorticoids down-regulated the expression of AnxA1 in CD4⁺ T cells (D'Acquisto et al., 2008a).

Annexin A1 in atherosclerosis

To date, only a few studies have explored the AnxA1 expression in atherosclerosis, despite its potential role in inflammation. In a human proteomics study of coronary arteries, the levels of AnxA1 were found to be increased in atherosclerotic tissue compared with non-atherosclerotic tissue (Bagnato et al., 2007). Subsequently, two studies reported that the expression of AnxA1 was higher in carotid plaques from asymptomatic compared with plaques from symptomatic patients (Cheuk and Cheng, 2011; Viiri et al., 2013). Interestingly, in a recent study using mouse models, AnxA1 was shown to reduce atherosclerosis by preventing the chemokine-induced recruitment of inflammatory cells (Drechsler et al., 2014).

AIMS

The overall aim of this thesis was to deepen the knowledge of pro- and anti-inflammatory mechanisms in CAD via phenotypic assessments of immune cell subsets, in particular CD56⁺ T cells, and exploration of AnxA1. The long-term goal is to reveal basic mechanisms that will lead to the development of biomarkers, which may be used for individualized treatment and monitoring.

Specific aims were

- to assess the expression of AnxA1 and GRs in neutrophils from patients and controls and its association with neutrophil activation status, and evaluate the effects of AnxA1 on neutrophil activation *ex vivo*.
- to investigate whether the expression of CD56 on T cell subsets differed between patients and controls, and further characterize the cytokine profile of CD56⁺ and CD56⁻ T cells.
- to characterize the expression pattern of AnxA1 in CD56⁺ and CD56⁻ T cell subsets from patients and controls, and also evaluate the effects of dexamethasone and AnxA1 on CD56⁺ and CD56⁻ T cells *in vitro*.
- to measure the expression of AnxA1 mRNA and protein in PBMCs from patients and controls, and evaluate its association with LPS-induced cytokine secretion and glucocorticoid sensitivity *ex vivo*.

METHODOLOGICAL CONSIDERATIONS

Study populations

All studies were approved by the Ethical Review Board of Linköping University, and conducted in accordance with the ethical guidelines of Declaration of Helsinki. Informed consent was obtained from all study participants (paper I-IV). Characteristics of subjects included in the different studies are summarized in Table II. All patients were recruited at the Department of Cardiology, University Hospital, Linköping, Sweden. Exclusion criteria: severe heart failure, immunologic disorders, neoplasm disease, evidence of acute or recent (< 2 months) infection, recent major trauma, surgery or revascularization procedure, drug or alcohol abuse, poor mental function, or treatment with immunosuppressive or anti-inflammatory agents (except low dose aspirin).

All control subjects were randomly selected from a population-based register representing the hospital recruitment area. Control subjects were included if they were anamnesticallly healthy, with normal routine laboratory test results, no clinical signs or history of CAD, or peripheral artery disease. The use of statins or antihypertensive drugs were allowed for primary prevention. In addition, in paper III, 11 anonymous blood donors were included from the Blood bank at Linköping University Hospital, Linköping, Sweden. They had no symptoms of disease, normal blood pressure, were drug-free and seronegative for HIV, hepatitis B and C, and syphilis.

Blood samples were always drawn between 8 and 12 A.M. in all studies.

Table II. Summary of study participants included in the four different papers of the thesis.

	Paper I	Paper II	Paper III	Paper IV
ACS, n		30	28	10
Age, years		69 (60-78)	68 (61-73)	68 (61-73)
Females, n (%)		8 (27)	8 (29)	3 (30)
Statins, n (%)		9 (30)	8 (29)	4 (40)
Stable CAD, n	30	34	57	57
Age, years	63 ($\pm$ 5)	63 (58-71)	66 (61-71)	66 (61-71)
Female, n (%)	5 (25)	6 (18)	13 (23)	13 (23)
Statins, n (%)	30 (100)	28 (82)	56 (98)	56 (98)
Healthy controls, n	30	36	52	41
Age, years	63 ($\pm$ 5)	63 (58-73)	66 (57-72)	67 (66-72)
Female, n (%)	5 (25)	9 (25)	16 (31)	11 (27)
Statins, n (%)	3 (10)	0	4 (8)	4 (10)
Study participants, n	60	100	137	108

Paper I

In the first study, 30 patients with stable CAD and 30 age and sex matched controls were recruited in 2005-2006. Individuals ≤ 70 years old were included. Inclusion criteria for patients were effort-related angina in accordance with the Canadian Cardiovascular Society functional classes I and II without any worsening of symptoms during the last 6 months. In each experiment, venous peripheral blood was drawn from one patient and his/her matched control in the morning after a 12 h fast and the samples were treated in pairs. Examiners were always blinded to the samples.

Paper II

In the second study, 30 patients with ACS, 34 patients with SA, and 36 healthy controls (with equivalent age and sex distribution) were included during the years 2006-2010. We initially aimed to include patients ≤ 75 years old but in some cases, elderly individuals were included. Patients were followed

longitudinally, and samples were collected after 3 months from 16 ACS and 28 SA patients, and after 12 months from 15 ACS and 28 SA patients. All patients underwent coronary angiography at day 1, and blood samples were collected prior to coronary angiography, within 24 h from admission in ACS patients.

Inclusion criteria for ACS patients were diagnosis of unstable angina/non-ST elevation myocardial infarction, with the diagnosis based on typical ECG-changes (ST-T segment depression and/or T-wave inversion) and/or elevated troponins. Inclusion criteria for SA patients were referral for elective coronary angiography due to effort angina class II or III in accordance with Canadian Cardiovascular Society Classification and without any worsening of symptoms the latest 3 months.

Paper III-IV

In the third and fourth study, all individuals were ≤ 75 years old. In total, 41 healthy subjects, 28 ACS patients, and 57 post-ACS patients were included 2011-2014. In paper IV, only samples from 10 of the 28 ACS patients were assessed. Moreover, in paper III, 11 anonymous blood donors were included.

Inclusion criteria for the patients with acute or stable conditions of CAD were ACS defined as a diagnosis of non-ST elevation myocardial infarction based on typical ECG changes (ST-T segment depression and/or T-wave inversion) and elevated troponins. Blood was collected within 24 h from admission, always prior to coronary angiography in ACS patients. In post-ACS patients, blood was collected 6-12 months after the acute event.

Laboratory methods

The laboratory methods are described in detail in the Material and method section of each respective paper included in the thesis, and summarized below. Thereafter, the general principles for the most prominent methods are described. Finally, supplementary methods that are not described in any of the papers are presented.

Summary of methods used in Paper I-IV

In paper I, we assessed the expression of AnxA1, FPRL-1 (FPR2/ALX), GR-total, GR- α , GR- β , and CD18 (unstimulated + LTB₄-stimulated samples) by one color whole blood flow cytometry in neutrophils gated by forward scatter (FSC) and side scatter (SSC) characteristics. We further evaluated recombinant AnxA1 (rAnxA1) influence on LTB₄-induced ROS production in isolated PMN. Morning and evening salivary cortisol was also measured during three consecutive days.

In paper II, CD8⁺CD56⁺ and CD8⁺CD56⁻ T cells were characterized regarding cell specific markers, numbers and proportions, markers for apoptosis, activation, and immunosenescence by 6- or 7-color whole blood flow cytometry. After whole blood stimulation, cells were stained both for surface markers (including the early activation marker CD69) and intracellularly for either IFN- γ , IL-10, IL-13, IL-17A or TNF. Furthermore, CRP was measured in serum samples, and IL-6 and IL-15 were analyzed in plasma samples with enzyme-linked immunosorbent assay (ELISA).

In paper III, we further characterized CD56⁺ and CD56⁻ T cells, and cell surface-associated as well as total AnxA1 was analyzed with multicolor whole blood flow cytometry. Peripheral blood mononuclear cells (PBMC) were isolated, and CD3⁺CD56⁺ and CD3⁺CD56⁻ cells were sorted using magnetic beads isolation. The isolated CD56⁺ and CD56⁻ T cells were stimulated with phorbol 12-myristate 13-acetate (PMA) in combination with different doses of the synthetic glucocorticoid dexamethasone, as well as rAnxA1. Secreted IFN- γ , IL-10, TNF, was measured in cell supernatants with Luminex, and cells were analyzed both for mRNA and protein (cell surface-associated and total) levels

of AnxA1 with reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and multicolor flow cytometry.

In paper IV, the expression of cell surface-associated and total AnxA1 protein was analyzed in monocytes, CD4⁺ T cells, CD8⁺ T cells and NK cells with multicolor whole blood flow cytometry. In freshly isolated PBMC, AnxA1 mRNA levels were analyzed with RT-qPCR. Isolated PBMC were also stimulated with LPS in combination with different doses of dexamethasone. Thereafter, stimulated cells were analyzed for AnxA1 mRNA with RT-qPCR, and IL-1 β , IL-6, IL-10, and TNF were measured in cell supernatants with Luminex. Moreover, IL-6 and IL-1 receptor antagonist (Ra) were analyzed in plasma using ELISA and Luminex, respectively.

Flow cytometry

General principles

Flow cytometry is a quantitative method for analyzing different particles (*e.g.* cells) in suspension, simultaneously measuring multiple parameters on a single cell level for many thousand cells per second. Cells labeled with fluorochrome-conjugated antibodies directed towards the antigen of interest, are forced into a single cell stream due to hydrodynamic focusing created by the carrier liquid/sheath fluid. Cells then individually pass a laser beam, during which light is scattered and fluorochromes are excited to a higher energy state emitting light of a wavelength specific for each fluorochrome. The scattered and emitted light is collected via optics that direct the light into a series of filters and dichroic mirrors, isolating specific wavelength bands that are detected by photomultiplier tubes (PMTs). The PMTs convert the light signals into electrical pulses and further digitalize the data for computer analyses (Figure 3).

Figure 3. A summary of the general principles of flow cytometry. See text for explanation

The forward-scattered light (FSC) is proportional to the cell size, and the side-scattered light (SSC) is relative to internal complexity/ granularity of the cell. By plotting FCS against SSC, cell types in a heterogeneous cell population (e.g. leukocytes) can be differentiated. The intensity of the fluorescence signal corresponds to the number of fluorochromes on the single cell, and is presented as median (or mean) fluorescence intensity (MFI).). In addition, cells that are labeled by the fluorochromes can be distinguished from cells that are unlabeled.

Some fluorochromes can have broad relative emission spectra, or several emission peaks, which results in leakage/spill-over to several detectors. When designing a panel of fluorochromes for multicolor flow cytometry, choosing fluorochromes with the least spectral overlap is desirable. However, to totally circumvent spill-over in multicolor flow cytometry is almost impossible, even though the constant introduction of new fluorochromes and the development of instruments with more lasers and detectors make it easier and easier. Also, the spill-over can be quantified, and manually or automatically compensated for by subtracting the leakage from the “wrong” detector.

Analysis of flow cytometry data

Flow cytometry data are analyzed in specific analysis software, and since this is manually made, it is also a weak point of the method. The data can be displayed in histograms (showing one parameter at a time) or two-dimensional dot-plots (showing two parameters at a time). In the histograms, regions can be inserted to identify specific groups of cells. In dot-plots, the region of interest is called gate, and can be in the shape of a rectangle, ellipse or a freely drawn area. Gated cells can be displayed in a new dot plot, or in a histogram, enabling analysis of multiple parameters and identification of subpopulations of cells. Most commonly, FSC and SSC data are showed on a linear scale, and the fluorescence intensity on a logarithmic scale. The protein expression is most commonly presented as a percentage of labeled (positive) cells, but quite often also as MFI.

When defining cells as positive or negative for a marker of interest, isotype controls and fluorescence minus one (FMO) are helpful tools. Isotype controls are antibodies directed towards irrelevant antigens, but of the same isotype and labeled with the same fluorochrome as the fluorescent antibodies used in the panel. The isotype control will give the unspecific background staining in a cell population, which is used for setting the lower limit of the gate for truly positive stained cells. When polyclonal antibodies are used, the fluorescent conjugated secondary antibody can be used alone to define the background staining. In flow cytometry panels where multiple colors are used, single isotype controls are not appropriate since the limits of truly positive stained cells also can be influenced by the total fluorescence in all channels. Here the FMOs are used instead. The FMO is exactly what the name says; the combination of all of the fluorochromes in a panel except one. Normally, the excluded fluorochrome (the “minus one”) is replaced by the corresponding isotype. The limits of the gates are then set after “the one”. FMOs are especially helpful when the antigen of interest is weakly expressed, or if it is continuously expressed, *i.e.* when the positive population is hard to discriminate from the negative population. In many cases, in particular for lymphocytes, the contour of positive and negative populations can be used to set the gates.

Enzyme-linked immunosorbent assay (ELISA)

The ELISA technique uses the principle of antigens binding to specific antibodies for detecting or quantifying small amounts of antigens (*e.g.* proteins, like cytokines) or antibodies in liquids (such as serum, plasma or cell supernatants). The principle for the sandwich ELISA used in paper II and IV, is that the bottom of a well in a microtiter plate is coated with the capture antibody. The sample containing the protein of interest is then added, whereafter the horseradish peroxidase (HRP)-conjugated detection antibody is added capturing the protein in the middle like a sandwich. The two antibodies used have to bind to different epitopes of the antigen. Finally a detection solution containing a substrate is added, which is cleaved by HRP into a colored or chemiluminescent product. The absorbance or the emitted light is

proportional to the levels of protein, and quantified using a standard curve of known protein concentrations.

Luminex

Luminex is a multiplex bead-based immunoassay, based on the same immunological principle as ELISA with antibodies and antigens, but may also be considered as a mixture of the ELISA and flow cytometry technology. By using Luminex, up to 500 analytes can theoretically be detected in a single sample using less sample volume than for ELISA. However, in practice, between one and 30 analytes are usually used at the same time.

The principle of the method is that the capture antibody is coated to polystyrene or magnetic beads, filled with a specific mixture of fluorescent dyes. Since the color-mixture of each bead is unique, differently dyed beads coated with different antibodies can be mixed together in the same well, thus enabling detection of multiple analytes in a single sample. Sample matrix might be plasma, serum or cell supernatants. If the beads are magnetic, a microplate is used, but if the beads are polystyrene, a filter plate has to be used since the washing procedure is different compared with ELISA. The magnetic beads are washed using a magnet underneath the plate, to keep the beads stuck in the wells during the washing steps. The polystyrene beads are washed using a vacuum manifold device, removing the liquid through the filter bottom, leaving the beads in the wells.

In the next step, the biotin conjugated detection antibodies are added to the beads with the bound analytes, whereafter streptavidin-PE is added to the wells. The beads are then read on a flow-based detection instrument with dual lasers. One of the lasers detects the bead, determining what analyte is being measured, and the other laser assess the PE fluorescence intensity, which is proportional to the amount of bound analyte. The concentration of the analyte can be calculated using a standard curve, with known concentrations of the analytes.

TaqMan reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

In TaqMan RT-qPCR, also called real-time RT-PCR, the expression of messenger RNA (mRNA) is assessed. After extraction, the mRNA is converted to complementary DNA (cDNA) by reverse transcription (RT) to complementary DNA (cDNA). The amount of cDNA is exponentially proportional to the amount of mRNA. In TaqMan RT-qPCR, probes contain a fluorescent reporter in the 5' end, and a quencher molecule in the 3' end. The quencher absorbs energy from the excited fluorochrome by fluorescence resonance energy transfer (FRET), resulting in silencing of the fluorochrome. During the amplification, the fluorescent reporters and quenchers are cleaved by Taq polymerase from the probes that bound to the cDNA, and the fluorescent reporters dissociate from the quenchers. With the increased distance between the quenchers and reporters, FRET is disrupted, and the fluorochrome starts to emit light. The initial amount of cDNA /mRNA can be calculated from the number of cycles required to reach cycle threshold (Ct). As a control for unequal loading of starting material, the amount of a house keeping gene with stable, constitutive expression (e.g. 18S reference RNA (rRNA)) is also assessed. The amount of cDNA/mRNA can be quantified relative to the rRNA and relatively to a calibrator using the $\Delta\Delta C_t$ method.

***In vitro* cell stimulation**

Several different stimuli have been used for *ex vivo/in vitro* cell stimulation in this thesis. The various methods used in the different papers are briefly summarized below.

In paper I, the intermediate chemoattractant LTB₄ was used for activation of neutrophils, both in whole blood and isolated PMNs, assessed by measurement of CD18 and ROS, respectively.

In paper II, the combination of PMA and ionomycin was used as stimulus for T cells in whole blood together with co-stimulatory antibodies against CD28/CD49d. The mitogen PMA directly activates protein kinase C, thereby acting downstream of receptor-mediated activation. The Ca²⁺ ionophore

ionomycin synergizes with PMA, enhancing the activation of protein kinase C. The protein secretion inhibitor Brefeldin A was added to the whole blood stimulation in order to allow the flow cytometric detection of intracellular cytokines in various T cell subsets. In paper III, PMA + ionomycin was used to induce cytokine secretion, from isolated CD56⁺ and CD56⁻ T cells. Moreover, mRNA and protein expression of AnxA1 was analyzed in stimulated cells in paper III.

In paper IV, the PAMP LPS, which is recognized by the PRR TLR4, was used as stimulus in isolated PBMCs. In particular, the cytokine secretion from monocytes is stimulated by LPS. IL-1 β , IL-6 and TNF are secreted at high levels within 4-8 h after LPS-stimulation, but IL-10 is not detectable until after 7 h, hence the longer incubation time of 19 h was chosen.

The effects of rAnxA1 protein and dexamethasone, a synthetic glucocorticoid, were investigated. In paper I, isolated neutrophils were pre-treated with rAnxA1 protein in order to study whether it affected LTB₄-induced ROS-production. In paper III, the effects of rAnxA1 and dexamethasone, alone or in combinations, on PMA-induced cytokine secretion from CD56⁺ and CD56⁻ T cells were explored. Isolated CD56⁺ and CD56⁻ T cells were incubated with dexamethasone at lower “physiological” doses (10⁻⁸ M) and higher “pharmacological” doses (10⁻⁷ M). Furthermore, the effects of dexamethasone on AnxA1 mRNA and protein expression in isolated CD56⁺ and CD56⁻ T cells, were assessed. In paper IV, the effects of dexamethasone on LPS-induced cytokine release, and expression of AnxA1 mRNA, from PBMCs were studied.

Supplementary methods

In study III, the expression of cell surface-associated and total AnxA1 protein was not only assessed in PBMC subsets, but also in neutrophils. Although the neutrophil data are not included in any of the papers of the thesis, they are presented in the Results and Discussion section of the thesis, and the method is described below.

Cell surface-associated and total expression of AnxA1 in neutrophils

The expression of AnxA1 protein in neutrophils was analyzed by flow cytometry in the same whole blood samples as described for the PBMC subpopulations in paper IV. Briefly, erythrocytes were lysed after surface antibodies staining. To detect cell surface-associated AnxA1, cells were first washed with phosphate-buffered saline (PBS) + 0.5% Fetal Bovine Serum (FBS), where after unspecific binding was blocked with goat serum and subsequently stained with rabbit anti-AnxA1. After washing, cells were stained with the secondary goat anti-rabbit F(ab')₂-PE, washed, and then permeabilized/fixed with Permeabilizing solution 2 (Perm 2). After centrifugation, cells were resuspended in 1% paraformaldehyde (PFA)/PBS and kept at 4°C in the dark until flow cytometry analysis. For detection of total AnxA1 protein, cells were first permeabilized/fixed with Perm 2, and after centrifugation, blocked with goat serum, before stained with rabbit anti-AnxA1. After washing, cells were incubated with secondary antibody (goat anti-rabbit F(ab')₂-PE), washed and finally resuspended in 1% PFA/PBS, and kept at 4°C in the dark until flow cytometry analysis.

As a control for background staining, samples without primary anti-AnxA1 were used, and samples were analyzed with Beckman Coulter Gallios. Data were analyzed, and granulocytes gated using FSC/SSC characteristics in combination with the exclusion of CD14⁺ cells, using the Kaluza software (version 1.2).

The assessments of AnxA1 expression in paper I and IV are not comparable due to major methodological differences. Most importantly, in study IV, both cell-surface associated and total AnxA1 expression was analyzed whereas only total expression of AnxA1 was studied in paper I. Furthermore, the protocols, including buffers, incubation times, and temperatures, differ significantly. In paper I, the protocol was designed specifically to study neutrophils. Even though the same antibodies were used in both studies, the F(ab')₂-fragment had different fluorochromes in the two studies; and in study IV, AnxA1 was also used in combination with other surface markers. Moreover, the samples were analyzed by using different instruments and software.

Statistical methods

In paper I, the data are presented as mean $\pm$ SD. Student's t test was used to compare differences in means for clinical and laboratory characteristics between patients and controls. Wilcoxon signed ranks test was used to compare differences between a patient and his/her matched control. Correlation analysis was performed using Spearman rank correlation test.

In paper II-IV, data are presented as median (inter-quartile range). Differences across three groups were analyzed with Kruskal-Wallis test, and Mann-Whitney U-test was used for between-group analyses. χ^2 test was used for nominal data. Differences between samples measured on 3 occasions were analyzed using Friedman test, while Wilcoxon signed ranks test was used for pair-wise comparisons. For correlation analyses Spearman's rank correlation method was used.

In paper II, multiple linear regression analysis was performed to assess the independent contribution of different factors to T cell changes. In paper III, data from the *in vitro* experiments were analyzed with paired samples t test.

In all studies, $p < 0.05$ was considered to be statistically significant. Statistical analyses were performed using SPSS version 15.0 (paper I), SPSS Inc., Chicago, IL, US, IBM SPSS Statistics version 19.0 (paper II), or version 22.0 (paper III-IV), IBM Corp., Armonk, NY, US.

RESULTS AND DISCUSSION

AnxA1 expression in neutrophils

Since a perturbed HPA-axis is known to be associated with chronic inflammatory conditions, and an association between dysregulated cortisol levels and inflammation was demonstrated in patients with CAD (Nijm et al., 2007), we wanted to investigate the impact of potentially dysregulated cortisol mechanisms on a cellular level, including expression of AnxA1 and GRs, in CAD patients and healthy controls. In paper I, we found that SA patients displayed a flattened diurnal cortisol slope, with increased evening levels of cortisol, compared with healthy controls (paper I, table 2), thus confirming our previous findings of a flattened diurnal cortisol slope in post-ACS patients (3 months after ACS)(Nijm et al., 2007). In line with previous results (Nijm et al., 2007), we also observed a significant correlation between evening levels of cortisol and CRP levels. When measuring the GRs in neutrophils, we found that they were ubiquitously expressed. However, GR-total as well as GR- α MFI levels were significantly lower in SA patients compared with their matched controls, whereas the expression of GR- β did not show any significant difference between patients and controls (paper I, figure 1). These findings suggest a state of cortisol desensitization of GRs in patients; resulting as a negative feedback due to high cortisol levels. As reported previously by Nijm *et al.* (Nijm et al., 2007), the total (24 h) cortisol output was increased in post-ACS patients compared with controls. Also, the up-regulation of GR- β in leukocytes has been associated with hypercortisolism, while the up-regulation of GR- α has been seen in individuals with hypocortisolism (Hagendorf et al., 2005). Furthermore, a reduction in total GR expression has been reported in chronic inflammatory diseases, like RA and systemic lupus erythematosus (SLE) (Li et al., 2010; Schlaghecke et al., 1992). In patients with SLE, GR- α expression was also negatively correlated with disease activity (Li et al., 2010). In the present study, we cannot conclude whether the decreased neutrophil expression of GR- α in CAD patients reflects a state of hypercortisolism, since we did not assess

the total cortisol output but instead laid focus on the diurnal variation by collecting morning and evening samples. However, a weak but significant inverse association between GR-total expression and morning cortisol levels was observed (paper I, table 3).

As glucocorticoids have been shown to induce AnxA1, we next measured the expression of AnxA1 protein in permeabilized neutrophils, *i.e.* the total expression of both intracellular and cell surface-associated AnxA1. AnxA1 was ubiquitously expressed, but the MFI levels were found to be significantly higher in neutrophils from SA patients compared with neutrophils from healthy controls (paper I, figure 2). Moreover, AnxA1 expression showed inverse correlation with both GR- α and GR-total expression (paper I, table 3). On the other hand, there was a positive correlation between AnxA1 and evening cortisol levels (paper I, table 3). Taken together, the findings of reduced expression of GR- α and increased expression of AnxA1 in SA patients may be compatible with an overexposure to cortisol. The results are also in line with previous findings by Mulla *et al.* (Mulla *et al.*, 2005), showing that the expression of AnxA1 in neutrophils reflected the levels of serum cortisol.

In paper I, we also assessed some markers of neutrophil activation represented by the basal and LTB₄-induced expression of the β_2 -integrin CD11b/CD18, as well as the LTB₄-induced ROS production. We found that the activation status of neutrophils from SA patients was similar to controls. Also, we evaluated the effects of AnxA1 in neutrophils, and found, somewhat unexpectedly, that exogenous rAnxA1 significantly suppressed the LTB₄-mediated ROS production from neutrophils in SA patients, but not in controls (paper I, figure 3). The results thus indicated a hypersensitivity to AnxA1 in SA patients compared with controls. There was a similar expression of the AnxA1 receptor FPR2/ALX, here called FPRL-1, in neutrophils from SA patients and controls (paper I), suggesting that the different response to rAnxA1 was independent of receptor expression. So far, we can only speculate on the reasons for the hyper-responsiveness to rAnxA1. It is likely to be the result of an over-activated HPA axis, but whether this *ex vivo* effect has any clinical relevance is unclear. One limitation is that we did not assess the effects of dexamethasone in these experiments.

A number of previous studies have shown differing results regarding neutrophil activation in CAD (Lindmark et al., 2001; Mehta et al., 1989; Nijm et al., 2005; Ott et al., 1996; Paulsson et al., 2007). In a previous study, we were not able to show that neutrophils from CAD patients were more prone to stimulation by IL-8 or LTB₄, than neutrophils from controls (Sarndahl et al., 2007). On the other hand, both ROS production in response to C3bi-opsonised yeast particles, and the neutrophils' inherent capacity to produce ROS, were significantly decreased in patients. However, in a more recent study, we showed that neutrophils from CAD patients were more prone to release MMP-9 upon stimulation with IL-8, compared with neutrophils from controls (Jonsson et al., 2011). The activation status of neutrophils in CAD patients remains to be clarified. The different results over the years may be due to a variety in *ex vivo* stimulation models. Also, differences in the use of cardiovascular drugs with potential effects on neutrophils, such as platelet inhibitors (aspirin, clopidogrel) and statins, may play a role (Egger et al., 2001; Guasti et al., 2006).

In the study cohort described in paper IV, including ACS patients, post-ACS patients and healthy controls, we measured both cell surface-associated and total AnxA1 protein expression in neutrophils. AnxA1 was ubiquitously expressed in neutrophils, both on the cell surface and intracellularly. No significant differences were seen in total MFI levels of AnxA1 protein between the three groups, ACS: 26 (21-30), post-ACS: 20 (17-26), controls: 22 (17-27), *p* for trend 0.200. However, when evaluating the cell surface-associated expression of AnxA1, we found that ACS patients expressed significantly higher amounts of AnxA1, compared with both post-ACS patients and controls, while cell surface levels were similar in the latter two groups (Figure 4). So, the results of total AnxA1 expression differed from those described in paper I. This may be due to considerable methodological differences as discussed in the Method section above. Furthermore, the SA patients in paper I may be regarded as a more heterogeneous group regarding CAD history. There are also several years between the collection of samples in the two cohorts, and since there has been a considerable reduction in MI deaths during the last decade, it is plausible that the post-ACS patients are under better treatment today. This is also supported by the fact that we in paper IV were unable to detect any significant

differences in evening cortisol levels between post-ACS patients and controls (paper IV, table I). However, we observed a correlation between cell surface AnxA1 expression and evening cortisol levels, but only in post-ACS patients, not in controls ($r = 0.303$, $p = 0.033$).

Figure 4. Expression of cell surface-associated AnxA1 protein in neutrophils from CAD patients and controls, analyzed by flow cytometry and presented as median fluorescence intensity (MFI). Trend across the groups $p = 0.005$. Between-group differences: *** $p < 0.001$. $N = 10$ ACS patients, 54 post-ACS patients, and 37 healthy control subjects.

The numbers of neutrophils in peripheral blood did not differ significantly between SA patients and controls in paper I (table 1), neither did it differ between post-ACS and controls described in paper IV ($3.7 (2.8 - 4.3) \times 10^3$ cells/ μL , and $3.2 (2.7 - 4.1) \times 10^3$ cells/ μL , respectively). As expected, the numbers of neutrophils were significantly increased in ACS patients ($5.1 (4.0 - 8.2) \times 10^3$ cells/ μL) compared with post-ACS patients and controls, $p = 0.011$ for trend. Elevated neutrophil count is a well-known characteristic of ACS patients and has also been shown to be a predictor of cardiovascular outcome (Guasti et al., 2011).

CD56⁺ T cells –functional phenotype and AnxA1 expression

Elevated numbers of CD56⁺ T cells have been reported in chronic inflammatory conditions, such as RA, sarcoidosis and Behcet's uveitis (Ahn et al., 2005; Katchar et al., 2005; Michel et al., 2007; Yu et al., 2004). Still, the role of this T cell subset in inflammation is not fully understood. We therefore wanted to investigate the CD56⁺ T cells and their characteristics, including cytokine production and AnxA1 expression, in CAD. As has been shown earlier (Ohkawa et al., 2001), the majority of CD56⁺ T cells in peripheral blood resided in the CD8⁺ population. In healthy subjects, 67 (54-82) % of the CD56⁺ T cells were single-positive for CD8, while 15 (9.9-24) % were single-positive for CD4. Only a minority of the CD56⁺ T cells were double-positive or double-negative for CD4 and CD8, 2.7 (1.2-5.1) % and 6.2 (3.6-14) %, respectively (paper III, table 1). The gating strategy for the CD56⁺ T cell subsets is shown in figure 5. We found significantly more CD8⁺CD56⁺ T cells, both assessed as total numbers and proportions of CD8⁺ T cells (paper II, table 3), in CAD patients compared with healthy controls, while the levels of CD4⁺CD56⁺ T cells did not differ. The increase was not due to higher absolute numbers of CD8⁺ T cells, since the total CD8⁺ T cell numbers were similar in patients and controls (paper III, table 3). The higher prevalence of CD8⁺CD56⁺ T cells was seen in both ACS and SA patients, and persisted over time, *i.e.* the increased levels of CD8⁺CD56⁺ T cells remained unchanged in both patient groups when measured on two more occasions over one year (paper III, figure 1). Hence, this phenomenon seemed to be independent of clinical presentation in CAD patients. In paper III, we confirmed that the proportions of CD56⁺ T cells within the lymphocyte population were significantly increased in post-ACS patients compared with controls, the majority residing in the CD8⁺ compartment (paper III, table I). However, in contrast to paper II, we were not able to demonstrate any significant differences in the proportions of CD8⁺CD56⁺ T cells within the CD8⁺ T cell population, when comparing ACS patients, post-ACS patients and controls in paper III.

Figure 5. The gating strategy of CD56⁺ T cells subsets (paper III).

In paper II, the expansion of CD8⁺CD56⁺ T cells in patients with CAD was neither associated with demographic or clinical variables such as age, sex, waist circumference, smoking, hypertension, diabetes, prior cardiac event, or number of coronary arteries with significant stenosis, nor was it correlated with statin treatment, levels of CRP, IL-6 or IL-15. As expected, we found a positive correlation between proportions of CD8⁺CD56⁺ T cells and cytomegalovirus (CMV) seropositivity ($r = 0.26$, $p = 0.011$), but CMV could not independently explain the higher levels of CD8⁺CD56⁺ T cells in CAD patients. CMV is a common, typically asymptomatic, life-long infection, which has been associated with increased cardiovascular risk (Simanek et al., 2011). Our findings in paper II are in line with a previous study by Romo *et al* (Romo et al., 2011), which also found increased proportions of CD56⁺ T cells in ACS patients compared with healthy subjects. Similar to us, they showed that the expansion of CD56⁺ T cells was independent of age, sex, traditional cardiovascular risk factors, and CMV seropositivity.

CMV has been suggested as an underlying cause of altered phenotype and functions in CD8⁺ T cells, predominantly in elderly people, as well as loss of CD28 in T cells, a phenomenon associated with immunosenescence (Koch et al., 2007). In an earlier study, our group found higher proportions of CD8⁺CD28⁻ T cells in SA patients, which was not only associated with CMV seropositivity but also with CAD *per se* (Jonasson et al., 2003). Furthermore, the gain of CD56 with a concomitant loss of CD28 on T cells have been described (Lemster et al., 2008; Michel et al., 2007; Tarazona et al., 2000). Accordingly, we found that the

majority of CD8⁺CD56⁺ T cells were CD28⁻ (paper II, table 4). Further, the loss of CD28 was much more pronounced in the CD8⁺CD56⁺ T cells compared with the CD8⁺CD56⁻ T cells, (71 (46-85) %, and 33 (19-57) %, respectively, $p < 0.001$. Moreover, the CD8⁺CD56⁺ T cells showed signs of increased activation (assessed by expression of the early activation marker CD69) and reduced apoptosis (detected by Annexin V) compared with their CD56⁻ counterparts. The reduced proportion of Annexin V⁺ cells among the CD8⁺CD56⁺ T cells may be a sign of a lower apoptotic rate of this cell subset, possibly explaining their accumulation in peripheral blood. In agreement with this, CD56⁺ T cells have been reported to express higher levels of the anti-apoptotic protein Bcl-2, further indicating that this phenotype is more resistant to apoptosis (Lemster et al., 2008).

Next, we wanted to characterize the cytokine profile of the CD56⁺ T cells. In paper III, we investigated the cytokine secretion in cell supernatants from CD56⁺ and CD56⁻ T cells, isolated from 11 healthy blood donors. After PMA-stimulation, the CD56⁺ T cells secreted significantly higher levels of IFN- γ and TNF compared with CD56⁻ cells, while the CD56⁻ T cells released about 30-fold higher levels of IL-10 compared with CD56⁺ T cells (paper III, figure 4). In paper II, we instead used a whole blood stimulation assay, and evaluated the intracellular cytokine expression in subgroups of study participants (9 ACS patients, 14 SA patients and 11 controls) with flow cytometry. After stimulation with PMA + ionomycin and anti-CD28/CD49d, and in presence of the protein secretion inhibitor Brefeldin A, the majority of both CD8⁺CD56⁺ T cells and CD8⁺CD56⁻ T cells were positive for IFN- γ and TNF, with no differences between ACS patients, SA patients or controls. The numbers of CD4⁺CD56⁺ T cells analyzed with flow cytometry were too few to allow a correct estimation. However, a similar pattern was seen, with the majority being positive for IFN- γ and TNF. In all 24 individuals, the proportions of IFN- γ ⁺ cells were significantly higher in the CD8⁺CD56⁺ T cell subset than in the CD8⁺CD56⁻ T cell subset (Figure 6). On the other hand, the proportions of cells expressing IL-10, IL-13 and IL-17A were low (<1%) in both CD8⁺CD56⁺ and CD8⁺CD56⁻ T cells, with no differences between CAD patients or controls. This was also the impression regarding the few CD4⁺CD56⁺ T cells. Our results regarding the cytokine profile of CD56⁺ T cells, are in agreement with previous studies reporting CD56⁺ T cells to be large producers of proinflammatory cytokines

such IFN- γ and TNF (Ahn et al., 2005; Katchar et al., 2005; Kelly-Rogers et al., 2006; Lemster et al., 2008; Ohkawa et al., 2001). A few previous studies have shown that CD56⁺ T cells are poor producers of IL-10 (Kelly-Rogers et al., 2006). In a clinical study by Shimamoto *et al.* (Shimamoto et al., 2007), CD56⁺ T cells were reduced in inflamed colonic mucosa of patients with ulcerative colitis, which led to the speculations that these cells might have anti-inflammatory effects. However, when stimulated *in vitro* with PMA, only a minor proportion (around 4 %) of the colonic CD56⁺ T cells from normal colonic mucosa expressed intracellular IL-10. We find it reasonable to propose that the accumulation of CD56⁺ T cells in peripheral blood of CAD patients represents a larger pool of cells with a pro-inflammatory phenotype. It remains to be clarified whether this is merely a marker of disease, or a factor that may aggravate the atherosclerotic process.

Figure 6. Proportions of IFN- γ ⁺CD8⁺ T cell subsets after PMA-stimulation, detected by intracellular flow cytometry. The negative population was set after unstimulated sample. Between-group differences: ** $p < 0.01$.

In paper III, we further characterized CD56⁺ and CD56⁻ T cells regarding AnxA1 expression. When analyzing AnxA1 expression in healthy subjects using whole blood flow cytometry, the protein was detected in the vast majority of T cells, however, the amounts differed significantly between T cell subsets. The CD8⁺ T cells were found to express higher levels of total AnxA1 protein than the CD4⁺ T cells (paper III, figure 1A). Moreover, the CD56⁺ T cells expressed more total AnxA1 protein than the CD56⁻ T cells (figure 1A), and correspondingly, AnxA1 mRNA was more abundantly expressed in the CD56⁺ T cells (paper III, figure 2). Also the cell surface-bound AnxA1 in whole blood appeared to be constitutively expressed on T cells but regarding this matter, CD4⁺ T cells expressed more cell surface-associated AnxA1 than CD8⁺ T cells (paper III, figure 1B). The cell surface-associated AnxA1 expression *in vivo* can be interpreted as an activation of the AnxA1 pathway, since AnxA1 exerts its autocrine and paracrine actions when translocated to the cell surface. However, when we assessed the cell surface-associated AnxA1 expression on isolated T cells after 6 h culture, we found that less than 10 % of the cells still expressed AnxA1. This might be an indication of AnxA1 being shed from the cell surface or secreted *in vitro*.

Hitherto, the knowledge about AnxA1 expression in human T cells has been limited to a few studies showing contradictory results. To the best of our knowledge, the AnxA1 expression has not been studied in CD56⁺ T cells. However, in an earlier study from 1995, Morand *et al.* (Morand et al., 1995) showed that a minority of CD4⁺ and CD8⁺ T cells expressed AnxA1, while a majority (85 %) of CD56⁺ lymphocytes (not identified with regard to CD3) expressed AnxA1. In a more recent study, Spurr *et al.* (Spurr et al., 2011) reported that all T cells stained positive for AnxA1, but that cell surface-associated AnxA1 was undetectable. The differing results may be partly explained by the fact that previous studies analyzed AnxA1 expression in isolated cells, a procedure that might have caused the loss of AnxA1 from the cell surface, and also included very few individuals, whereas we analyzed the protein expression in whole blood using a much larger sample size.

AnxA1 expression was also assessed in CD56⁺ and CD56⁻ T cell subsets from CAD patients, using whole blood flow cytometry. The total expression of AnxA1 protein did not differ between patients and controls, but there were

significant differences in cell surface-associated expression of AnxA1. The levels of surface AnxA1 in both CD8⁺CD56⁺ and CD8⁺CD56⁻ T cell subsets were higher in CAD patients compared with controls, and most pronounced in ACS patients (paper III, figure 3B). The differences in surface AnxA1 between patients and controls were more pronounced within the CD8⁺ T cell subset than within the CD4⁺ subset, thereby suggesting that the activation of the AnxA1 pathway in CAD patients might be more prominent in the CD8⁺CD56⁺ T cell fraction (paper III, figure 3A). There is sparse information regarding T cell expression of AnxA1 in human disease, but increased levels have been reported in autoimmune disease. In a previous study D'Acquisto *et al.* (D'Acquisto et al., 2007a) reported increased AnxA1 mRNA as well as increased total protein levels in isolated CD4⁺ T cells from 9 patients with active RA compared with healthy 5 controls.

The effects of AnxA1 on T cells are much less investigated than the effects on innate immune cells. Some experimental models have shown that deficiency of AnxA1 aggravates T cell-mediated inflammation (Gold et al., 1996; Kamal et al., 2001; Yang et al., 2004; Yang et al., 2013), whereas others have shown that deficiency of AnxA1 decreases the activation of T cells and promotes Th2 differentiation (D'Acquisto et al., 2007a; D'Acquisto et al., 2007b; Paschalidis et al., 2009). In paper III, we explored the effects of rAnxA1 protein and/or dexamethasone on PMA-induced cytokine secretion from isolated CD56⁺ and CD56⁻ T cells. Dexamethasone reduced the secretion of IFN- γ and TNF dose-dependently, while rAnxA1 alone had no effect (paper III, figure 5A-B). Only the higher dose of dexamethasone (10⁻⁷ M) inhibited IL-10 release from CD56⁻ T cells (paper III, figure 5C). When AnxA1 was combined with 10⁻⁷ M dexamethasone, the dexamethasone-induced suppression of IFN- γ and IL-10 secretion was abolished (paper III, figure 5A and C). Even if it can be argued that the effects of exogenous rAnxA1 in the presence or absence of dexamethasone *in vitro* may not be physiologically relevant, our results are in compliance with a previous study by D'Acquisto *et al.* (D'Acquisto et al., 2007a) showing that the dexamethasone-induced suppression of IL-2 secretion from CD4⁺ T cells was eliminated by adding AnxA1. The same authors showed in a subsequent study, that glucocorticoid treatment down-regulated AnxA1 mRNA expression in CD4⁺ T cells, both *in vivo* and *in vitro* (D'Acquisto et al., 2008a). On the other hand, in their earlier study from 1995, Morand *et al.* ,

(Morand et al., 1995) could not show that dexamethasone treatment for 24 h affected AnxA1 protein levels in human T cells. In line with this, we did not detect any significant changes in AnxA1 mRNA levels when we incubated isolated T cell subsets with 10^{-7} M dexamethasone for 6 h.

We can conclude that AnxA1 is predominantly expressed by T cells with a pro-inflammatory phenotype. Furthermore, the increased expression of cell surface-associated AnxA1 on T cells from ACS patients indicates that an increased activation of the AnxA1 pathway is associated with plaque destabilization and/or its consequences. Still, we cannot say whether this represents an attempt to counteract T cell activation. One intriguing possibility is that AnxA1 exerts pro-inflammatory effects in T cells, counteracting glucocorticoid effects, i.e. the total opposite of what is known to occur in innate immune cells. However, further studies are warranted to verify this hypothesis.

AnxA1 expression in PBMCs in relation to glucocorticoid sensitivity

As discussed above, the effect of AnxA1 on different leukocyte subsets is far from clarified. Due to the parsimonious information on AnxA1 expression in PBMC subsets in general, we wanted to study the expression pattern in various PBMC subsets, and also evaluate the possible role of AnxA1 as a marker for glucocorticoid sensitivity. When measuring the mRNA expression of AnxA1 in the total PBMC fraction from ACS patients, post-ACS patients and controls, we found significantly higher mRNA levels in the two groups of patients compared with controls (paper IV, figure 1). However, the expression was strikingly higher in the early phase of ACS (within 24h after the coronary event) compared with the post-ACS phase (6-12 months after the event). The detection of AnxA1 protein using whole blood flow cytometry analysis, showed results in line with previous findings (Morand et al., 1995; Spurr et al., 2011), *i.e.* the protein was to a greater extent expressed by monocytes than by T cell subsets and NK cells (paper IV, figure 2A and B). The increased expression in monocytes involved both total and cell surface-associated expression. The higher AnxA1 mRNA expression in ACS patients was reflected at the protein level, with a higher total AnxA1 expression in monocytes (figure 2A), and in particular, a higher amount

of AnxA1 protein on the cell surface of all PBMC subsets (figure 2B). Thus, signs of increased AnxA1 synthesis, as well as activation of the AnxA1 pathway involving the translocation of the protein to the cell surface, were evident in ACS patients.

As previously discussed, there are still limited numbers of studies exploring the presence or the role of AnxA1 in human atherosclerosis. Two studies of human carotid plaques suggested a plaque-stabilizing role for AnxA1 since the protein was more abundant in plaques from asymptomatic patients (Cheuk and Cheng, 2011; Viiri et al., 2013). Bagnato *et al.* (Bagnato et al., 2007) reported increased AnxA1 levels in the atherosclerotic areas of human coronary arteries. In the latter study, the protein was also immunohistochemically localized to macrophages and foam cells, findings that at a first glance might suggest AnxA1 as pro-inflammatory. However, the same authors demonstrated that AnxA1 was localized to an area of increased apoptosis, thus supporting a pro-resolving role of the protein and explaining its presence in foam cells as a result of efferocytosis. Our findings with the most prominent AnxA1 expression in PBMCs from the ACS patients, who exhibited the highest levels of systemic inflammation, may also seem contradictory. However, given an anti-inflammatory role of AnxA1, its increased expression in PBMCs under acute inflammatory conditions probably represents a rapid compensatory mechanism trying to counteract the pro-inflammatory response.

Glucocorticoids have in numerous studies been shown to induce both *de novo* synthesis of AnxA1, and to contribute to its translocation from the cytosol to the cell surface where the protein exerts its biological effects (Comera and Russo-Marie, 1995; McLeod and Bolton, 1995; McLeod et al., 1995; Solito et al., 2003). Moreover, there is convincing evidence that AnxA1 is a key mediator of anti-inflammatory effects in monocytes/macrophages, involving both rapid post-translational effects and transcriptional changes, thus promoting a stable anti-inflammatory phenotype (Lange et al., 2007; Strausbaugh and Rosen, 2001). Furthermore, in a randomized double-blind study, mild non-steroid anti-inflammatory therapy (diclofenac) given to overweight men for 9 days, resulted in increased expression of AnxA1 in PBMCs, thus revealing AnxA1 as a candidate marker for inflammatory modulation (van Erk et al., 2010). To further explore the significance of AnxA1 in human PBMCs, we studied its relation to

LPS-induced cytokine production and glucocorticoid sensitivity in 55 post-ACS patients and 31 controls. LPS induced significantly higher IL-6 secretion in PBMCs from patients compared with controls, whereas there was no difference in IL-10 secretion (paper IV, figure 3). It has been shown that human monocytes stimulated with LPS produce IL-10, which inhibits the synthesis of concomitantly secreted pro-inflammatory cytokines (de Waal Malefyt et al., 1991). Our results may thus be compatible with the presence of a pro-inflammatory cytokine profile of monocytes in post-ACS patients. An imbalance between pro- and anti-inflammatory cytokines in PBMCs has been reported in several previous studies of CAD patients (Calvert et al., 2011; Elsenberg et al., 2013; Waehre et al., 2002; Waehre et al., 2004).

Several animal models and human inflammatory diseases have shown that impaired responsiveness to glucocorticoids is associated with increased inflammatory activity (Quax et al., 2013). Also, it has been proposed in previous studies that the systemic inflammatory activity in patients with CAD may be associated with reduced sensitivity to glucocorticoids (Fantidis et al., 2002; Nijm et al., 2007). This could not be confirmed in paper IV. We rather found that PBMCs from post-ACS patients were slightly more sensitive to glucocorticoids *ex vivo*, compared with PBMCs from controls via the assessment on the ability of dexamethasone to suppress LPS-induced cytokine secretion (figure 4). Since glucocorticoids have been shown to induce the expression of AnxA1, a major aim was to evaluate whether the AnxA1 expression in PBMCs was related to glucocorticoid sensitivity. Somewhat unexpectedly, there were no correlations between the glucocorticoid sensitivity and the AnxA1 mRNA, or total AnxA1 protein levels in PBMCs. Instead, the glucocorticoid sensitivity *ex vivo* was found to correlate significantly with the expression of cell surface-associated AnxA1 protein in circulating monocytes. The data indicate that the quantity of AnxA1 located on the cell surface, rather than the total cellular content of the protein, is a marker for glucocorticoid sensitivity. In a previous study, Mulla *et al.* (Mulla et al., 2005) showed that AnxA1 protein expression in neutrophils, but not monocytes, was related to cortisol levels before, and 30 min after a standard corticotrophin test, proposing that AnxA1 could be used as a marker for endogenous glucocorticoid sensitivity. However, Mulla *et al.* did not measure cell surface-associated AnxA1 expression, which may explain the difference in

results regarding monocytes. In the present study, we also found a correlation between evening cortisol levels and expression of cell surface-associated AnxA1 in peripheral monocytes, which may further strengthen the suggestion that *in vivo* exposure for glucocorticoids could be reflected in extracellular AnxA1 expression. Moreover, evening cortisol levels correlated with circulating monocyte counts, but not with other leukocyte subset counts. We did not perform cortisol measurements over 3 consecutive days in the ACS patients, and can therefore only speculate that the elevated monocyte counts in ACS patients could have been associated with increased exposure to glucocorticoids.

Finally, we investigated if dexamethasone influenced the mRNA expression of AnxA1 in PBMCs from post-ACS patients and controls. When the higher dose of dexamethasone (10^{-7} M) was added to LPS-stimulated cells, it induced a significant increase in AnxA1 mRNA in both patients and controls (paper IV, figure 5). Our findings are in agreement with a study by Solito *et al.* (Solito *et al.*, 2003) demonstrating that 10^{-6} M dexamethasone induced an increase in AnxA1 mRNA in a human folliculostellate cell line. Furthermore, earlier *in vivo* studies have described substantially increased expression of AnxA1 protein in peripheral monocytes or PBMCs in healthy subjects after one single injection of hydrocortisone (Goulding *et al.*, 1990; Morand *et al.*, 1994).

In the post-ACS patients, the basal plasma levels of inflammatory markers, such as IL-6 and IL-1Ra, were as low as in the controls. Still, there was evidence for a low-grade subclinical pro-inflammatory activity in the patients, as assessed by LPS-induced IL-6 secretion from isolated PBMCs. An increased glucocorticoid activity might therefore have been expected. *Ex vivo*, the PBMCs in post-ACS patients were found to be at least as sensitive to glucocorticoids as were the PBMCs from controls. The increased AnxA1 mRNA levels in PBMCs from post-ACS patients indicated an increased glucocorticoid activity. Still, the levels of surface-bound AnxA1 were not increased which allows us to speculate about insufficient glucocorticoid actions and/or reduced glucocorticoid availability, and thereby an insufficient capacity to down-regulate inflammation.

CONCLUDING REMARKS

The AnxA1 protein was constitutively expressed in both neutrophils and PBMCs (lymphocytes and monocytes), not only intracellularly but also on the cell surface. However, there was a substantial variation in AnxA1 expression pattern among PBMC subsets, the protein being more abundant in monocytes than in lymphocytes. The AnxA1 expression also showed a large variation across T cell subsets, dependent on the expression of CD56. The CD56⁺ T cells, exhibiting a distinct pro-inflammatory phenotype, expressed significantly more AnxA1 than their CD56⁻ counterparts.

There were substantial differences in leukocyte AnxA1 expression pattern between CAD patients and healthy controls. Overall, the expression of AnxA1 was increased in patients.

In a cohort of patients with SA, the expression of total AnxA1 protein in neutrophils was higher than in controls. As might have been expected, this was not accompanied by altered neutrophil activation status in patients. However, the neutrophils from patients exhibited an enhanced anti-inflammatory response to rAnxA1 *ex vivo*, further emphasizing the potential of AnxA1 as an inhibitor of neutrophil activity. In a second cohort of CAD patients, recruited some years after the first cohort, no differences in total AnxA1 protein expression was observed between post-ACS patients and controls. Only patients with ACS showed an increase in cell surface-associated AnxA1 levels, which may illustrate a compensatory counteraction of an acute pro-inflammatory response.

CAD patients, independent of clinical presentation, had increased proportions of proinflammatory CD56⁺ T cells compared with controls, a phenomenon likely to represent immunological aging. In all T cell subsets, the expression of cell surface-associated AnxA1 was significantly increased in ACS patients whereas it tended to be increased in post-ACS patients. In addition, dexamethasone clearly reduced the *in vitro* secretion of pro-inflammatory cytokines from CD56⁺ T cells, whereas the addition of rAnxA1 abolished this effect. The *in vivo* findings indicate that the AnxA1 pathway is more activated

in T cells from CAD patients. However, the precise role of AnxA1 in T cells remains an enigma. Further investigations into this topic are required, especially since the potential use of AnxA1 as an anti-inflammatory drug is discussed.

In PBMCs, the mRNA levels of AnxA1 were increased in ACS and post-ACS patients, particularly in ACS patients, compared with controls. Correspondingly, the monocyte fraction in ACS patients, but not in post-ACS patients, exhibited increased AnxA1 protein levels, both totally and on the cell surface. T cells as well as NK cells in ACS patients expressed more AnxA1 protein on the cell surface, while only a tendency was observed in post-ACS patients. The expression of cell surface-associated AnxA1 in monocytes correlated with the glucocorticoid sensitivity of PBMCs *ex vivo*. Moreover, *ex vivo* assays suggested that PBMCs from post-ACS patients were even more susceptible to inflammatory stimulation compared with PBMCs from controls. Since the monocyte expression of cell surface-associated AnxA1 was similar in the two groups, it can be speculated that the glucocorticoid actions and/or availability in post-ACS patients were insufficient. One plausible mechanism behind reduced cortisol availability is blunted or impaired cortisol response to daily stressors. Previous studies have shown that post-ACS patients display a blunted cortisol response when exposed to physical or psychological stress (Nijm et al., 2007).

A wide range of soluble markers are available today for the detection of inflammatory activity. However, it still remains a challenge to measure the modulation of inflammation. We believe that expression of cell surface-associated AnxA1, as a marker of glucocorticoid sensitivity, is a promising candidate tool that, however, needs to be further investigated in larger cohorts and intervention trials.

ACKNOWLEDGEMENTS

This thesis would not have been possible to complete without the help and support from a lot of people, and I would like to express my sincere gratitude to everyone who in different ways have been involved with me and in my thesis over the years. I would especially like to thank:

All the **study participants**, without whom there would definitely not have been any thesis!

Lena Jonasson, my main supervisor, for giving me the opportunity to do a PhD, for your trust in me and for giving me the freedom to develop and being independent during these years. Your never ending optimism and enthusiasm is most inspiring! Thank you for always being available, whenever I have needed, always ready to kindly taking your precious time, and always being a stable rock to lean on! Your mind is a fantastic never ending source of ideas, sometimes a bit too fast for me to follow though. Your instinctive feel regarding projects, experiments and manuscripts is amazing! I will always admire your energy and your persistence! Thank you also for your guidance to the good and picturesque things in life in general, such as finding a nice little café or restaurant in every town we have visited together!

Eva Särndahl, my co-supervisor, for taking me under your wings and introducing me to science. It has been 10 years now since you gave me the opportunity to start doing research in your lab. Thank you for being such a brave and wise supervisor during my early years as a bachelor and master student, for giving me the trust to develop, making mistakes, and figuring out how to do things on my own in the lab! I will always be grateful to you for including me as a bachelor student in the MIMMI-II project, for introducing me to Lena. Thank you also for introducing me to neutrophils, AnxA1 and for giving me my first flow cytometry project so that I had to learn this fantastic method!

Jan Ernerudh, my co-supervisor, for sharing all your knowledge in the immunology-field. Thank you for always being an optimist, looking at the bright side of things, always staying calm, and kindly giving of your valuable

time. Your wise advices regarding T cells and flow cytometry have been invaluable!

Wei Li, my former supervisor and roommate, for your kindness and support during my first year as a PhD student, for introducing me to the field of apoptosis and letting me deepen my knowledge of flow cytometry.

Anna Lundberg, our principal research engineer and my closest colleague, for all your work you have put in to our studies! You have been invaluable to me! Since you came to our group, you have been the perfect spider in the web, keeping track on everyone and everything. Thank you for sharing both ups and downs in the lab, for always giving good advice and always being such a support! You have not only been the best colleague, but also become a dear friend! I hope we can continue celebrating various festivities together with you, **Micke** and your lovely boys, and this summer you really have to find the way the 7 km from Skärpingen to Penningtorp!

Simon Jönsson, my PhD-student colleague and co-author, for all your work with the MIMMI-III study. Thank you also for being such a fun and patient co-worker, and always on my side! It has been great always having someone more tired in the mornings and more last-minute than me. Thanks for always sharing your unique ideas regarding food, training and politics! I gave you the opportunity to finish your PhD first with my parental leave, but as the gentleman you are you still let me go first. I wish you all the best and good luck the week after me!

Olivia Forsberg, our research engineer, for the work you have put into paper III and IV. I'm so happy that we found you, and you wanted to work in our group! You have been a true asset in the lab, always a happy and considerate colleague.

Rosanna Chung, our post-doc, for being the most positive and curious colleague, full of energy! I really admire your intelligence and your ability to learn flow cytometry so fast! Thank you so much, again, for reading my thesis!

Karin Backteman, co-author, for all your work with the CorC study! Thank you for your patience and kindness, for always taking time for my questions and sharing all your knowledge and experience regarding flow cytometry! I really appreciate your sense of humor, and you sharing your escargots in Paris!

Carina Andersson, for all the lab work you did in the MIMMI-III study, and for always kindly answering all my questions and emails!

Ylva Lindegårdh, our former lab technician/biomedical scientist, for all your meticulous work in the MIMMI-II and CorC studies. Thank you also for taking such good care of both me and the lab during my first years working for Lena, always being a happy, kind and considerate colleague! I miss you a lot!

Johnny Nijm, co-author, for being such nice company in the lab during the MIMMI-II study and for treating me as your equal colleague even though I was just a bachelor student!

Prof. Mauro Perretti and **Prof. Chris Reutelingsperger** for good collaboration and providing me with AnxA1.

Tony Forslund, co-author, for introducing me to flow cytometry and good collaboration during the MIMMI-II study.

Aleksander Szymanowski, fellow PhD student in our group, for your contributions to the CorC study, interest in my work, and for nice times during congresses and courses.

Eva Hellqvist, **Chamilly Evaldsson** and **Kila Hasib**, our former post docs, and co-workers for your time and work in our group, especially your contribution to the MIMMI-III study.

Mona Börjesson, **Anneli Raschperger**, **Elisabeth Logander**, **Elisabet Kindberg**, **Christina Svensson**, **Lennart Nilsson**, **Helen Zachrisson**, **Per Leandersson**, and staff at the **Seldinger** unit for your help and work in the MIMMI-II, CorC, and MIMMI-III studies.

All the students we have had in the lab during the years, for your contributions. Especially to **Jonathan**, **Martin**, **Lida**, and **Johanna**.

Former colleagues at the Division of Medical Microbiology and Cell Biology, floor 12 for making my time during the MIMMI-II study so pleasant, especially to **Olle Stendahl**, **Maria Lerm**, **Veronika Patcha Brodin**, **Robert Blomgran** and **Deepti Verma**.

The former head of the Division of Cardiovascular Medicine **Ulf Dahlström**, for all the work you did for our division and for showing interest in my work.

Former and present heads of the Division of Drug research (previously Pharmacology) **Rolf Andersson, Karin Persson, Staffan Hägg, and Mårten Segelmark** for showing interests in my research and providing the facilities.

Ingela Jakobsson, Britt Sigfridsson and Erika Bjärehed for help with blood sampling.

Florence Sjögren for all your help during the years, and for sharing your great knowledge of flow cytometry!

My fellow PhD students over the years, especially **Hanna Kälvegren, Ann-Charlotte Svensson, Caroline Brommesson, Andreas Eriksson, Liza Ljungberg, and Hanna Björck**. Thank you for so kindly embracing me when we came to the division of Pharmacology! Thanks for all the help you have given me in different ways and the great times we have shared, I miss them and you who have left! Thank you also to **Anna Jönsson, Karin Skoglund, Louise Carlsson, Svante Vikingsson, Michaela Tjäderborn, Ingrid Jakobsen Falk, Daniel Söderberg, Anna Svedberg, Anna Zimdahl Kahlin, Niklas Björn, Sandeep Koppal and Zaheer Ali** for all the nice talks during lunches and coffee breaks. A special thank you to my roommates **Emina Vorkapic, Therese Gustafsson, Renate Slind Olsen** for putting up with me and for sharing our nice and warm room!

My former and present room neighbors, **Torbjörn Bengtsson, Magnus Grenegård, Dick Wågsäter** for all the nice talks and interest you have shown in me and my work!

The administrators who have been invaluable during the years, **Anita Thunberg, Yvonne Hallingström, Elin Wistrand, Erika Lindén, Madeleine Öhrlin and Malin Strand!** You have always made me feel welcome with my questions and helped me whenever I needed!

All my nice colleagues at Division of Drug Research, for creating such a pleasant work place! A special thanks to **Eva Lindström, Siv Nilsson, Per Whiss, Ingrid Persson, Marja Tjädermo, Ingrid Hurtig, Iréne Rydberg, and Lillemor Fransson** for making me feel welcome when we first arrived even though I belonged to the “wrong” division. Thank you also to my MedBi-mother **Susanne Andersson** for always caring for me and having time to listen to me whenever I have needed!

I am also very lucky to have a large network of friends, relatives and family outside work, whom I would like to take the opportunity to thank for being in my life!

Emma Börgheson, min kära vän ända sedan tiden då vi började i Evas lab. Först ett jättestort tack för att du trots ditt alldeles fullspäckade schema ändå fann tid att göra de fina bilderna åt mig till min avhandling! Tack också att du är mitt ständiga bollplank och för språklig hjälp! Det är för mig ovärderligt att ha någon som du som man alltid kan ringa till när man behöver bitcha av sig om stort som smått, någon som är omtänksam men ärlig och som förstår precis hur det är att vara i den här världen. Du är en klart lysande stjärna och du kommer att nå mycket långt i forskningsvärlden, jag är fantastiskt stolt över att få vara din vän! Tack också till din underbara familj, till **Arne**, **Gerd** och **Anna** med familj, som alltid även tar så väl hand om mig/oss när jag/vi kommer på besök!

Min relativt nyfunna vän **Anna F Söderström** som så generöst och självklart ställde upp och hjälpte mig med det fina omslaget till min avhandling! Tusen, tusen tack! Jag är så otroligt glad och stolt över det! Tack också för att jag får följa med på dina loppis-rundor, vi fyndar så mycket bra ihop du och jag! Vi är otroligt tacksamma för att du, **Johan** och **Otto** finns så nära oss, närhelst vi behöver ut och rasta barnen. Tack också för alla diskussioner kring högt och lågt rörande våra söner, födda dagarna efter varandra!

Mina MedBi-vänner **Helena Enocsson**, **Lotta Agholme**, **Anna Angel** och **Pernilla Eliasson**, för att ni dragit iväg mig från jobbet för trevliga luncher och fikor. Lotta, Anna och Pernilla med **era respektive** också för att ni dragit med mig och Johan på diverse upptåg, som fantastiska alpskidresor, och för att ni förser oss med fint kött!

Våra gemensamma vänner **Richard Mårtensson**, **Julia Malmberg** och er **Fiona** för att ni är sådana fina människor och vill semestra med oss lite nu och då. Nu ska vi försöka bli bättre på att återgälda era besök och komma upp till Falun snart!

Mina fina gymnasievänner **Louise Pettersson**, **Anna-Maria Valegård**, **Erika Karmegren**, **Agnes Ljunge**, och **Jenny Palmberg** för att ni fortfarande finns där

och för att vi har så trevligt ihop! Jag tänker på er mycket oftare än jag ger väsen av. Nu får vi se till att få lite bättre kontinuitet i våra träffar!

Mina kära barndomsvänner **Karin Sundberg** och **Lena Helldahl**, samt även **Louise**, ni betyder fortfarande otroligt mycket för mig även om vi inte alls hinner ses så mycket som jag skulle vilja! Tänk att ha sådana som ni som funnits med en i hela livet, bokstavligt talat! Tack för allt roligt vi hittat på genom åren, som "post, bank och försäkringskassa", eller något av våra berömda framträdanden, och för att ni än idag står ut med mig! Dessutom har ni ju fantastiskt trevliga äkta män i **Ante**, **Chrille** och **Martin** och underbara tjejer, så det är alltid en fröjd att få umgås med er! Ett tack också till era föräldrar och familjer **Tillbergs**, **Carlssons** och **Karlssons** för allt ni gjort för mig genom åren!

Min stora släkt med alla mostrar, fastrar, farbror, med respektive, alla mina trevliga kusiner med respektive och mina söta kusinbarn och kusinbarnbarn. Tack för att ni alltid finns där, alla trevliga kalas vi har, att ni är så enkla och härliga att umgås med och inte minst för att ni visar intresse för mig och mitt konstiga jobb! Ett speciellt tack till min älskade faster **Anita**, som förser mig med mat och hembakat bröd, för att du sticker sjalar, tröjor, handledsvärmare och sockor åt mig så jag slipper frysa! Tack också för allt engagemang du och **Kjell** lagt ner på mig genom åren och för allt ni gör för Theodor! I år ska vi se till att komma upp till "Pensionat Muskö", det var alldeles för länge sen nu!

Min svärfamilj **Katarina**, **Rolf**, **Marie**, **Martin**; **Arvid** och **Elin** för att ni så självklart finns i våra liv! Tack för all hjälp vi fått genom åren, att vi alltid är välkomna, allt gott ni förser oss med, och inte minst till barnen som är så fina med Theodor! Tack för att vi alltid är välkomna även i **Gärdemans**-familjen! Ett alldeles extra stort tack till farmor och farfar för allt ni gör för Theodor, för att ni rycker ut och hämtar på förskolan och barnvaktar närhelst vi behöver! Till **Emma**, **Susanne** och **Magnus**, jag tänker på er ofta!

Mina fina föräldrar **Ingrid** och **Lennart**, utan er hade ju detta aldrig gått, det hoppas jag ni förstår! Ni har alltid stöttat och peppat mig, ställt upp och engagerat er i allt jag gjort, kört oändligt antal mil för att skjutsa till skidtävling eller köra flyttlass. Tack för allt ni gjort och gör för mig!! Tack för att vi får komma till er både i Åtvid och i Penningtorp närhelst vi önskar! Tack för att ni göder oss med både mat och kärlek, hjälper oss med allt möjligt praktiskt och

inte minst för att ni tar så stort ansvar för Theodor! Tack för att ni med kort varsel kunnat rycka ut även när han varit sjuk och det kört ihop sig för oss! Ni är ovärderliga för oss!

Min älskade, bästa, finaste lillasyster **Lydia**, för att du är du och för att jag får vara jag! Du betyder så oändligt mycket för mig!! Tack för allt du gjort och gör för mig, både genom att piffa upp mig och min garderob och ta hand om min själ! Din omtänksamhet är makalös! Tack för att du alltid står på min sida, för att du alltid stöttar, peppar och hejar på din nördiga storasyster! Ett stort tack också till din fina man **Erik**, och till er underbara dotter, mosters älskade solstråle **Rut**, för att ni finns i våra liv! Jag vill också tacka din gulliga svärfamilj **Ersbergs** för all omtänksamhet även för mig och min familj!

Johan, min klippa och min följeslagare genom livet. Tack för all kärlek, allt stöd och för att vi utgör ett så bra team du och jag! Ett särskilt tack också för att du ställer upp på detta, för att du prioriterat ner i princip allt eget i ditt liv och istället tagit hand om all markservice och huvudansvaret för vår son under den här perioden. Och för att du även hunnit ta hand om mig! Tack för att du finns, och för att du står ut med mig! Älskar dig mest av allt!

Theodor, min allra finaste skatt, min tågtokiga lilla tigerunge. Tack för din villkorslösa kärlek, för allt du lär mig om livet och för att du noggrant påpekar när jag har fel! Älskar dig hela dagen! Uggå mugga!

This thesis was supported by grants from the Swedish Research Council, the Swedish Heart-Lung Foundation, Linköping University Hospital Research Fund, Lions Research Fund, and the County Council of Östergötland, Sweden.

REFERENCES

- Aardal, E., and Holm, A.C. (1995). Cortisol in saliva--reference ranges and relation to cortisol in serum. *European journal of clinical chemistry and clinical biochemistry : journal of the Forum of European Clinical Chemistry Societies* 33, 927-932.
- Abbas, A.K., Lichtman, A.H., and Pillai, S. (2014). *Basic immunology : functions and disorders of the immune system*, Fourth edition. edn (Philadelphia, PA: Elsevier/Saunders).
- Ahn, J.K., Chung, H., Lee, D.-s., Yu, Y.S., and Yu, H.G. (2005). CD8brightCD56+ T Cells Are Cytotoxic Effectors in Patients with Active Behçet's Uveitis. *The Journal of Immunology* 175, 6133-6142.
- Ait-Oufella, H., Sage, A.P., Mallat, Z., and Tedgui, A. (2014). Adaptive (T and B cells) immunity and control by dendritic cells in atherosclerosis. *Circ Res* 114, 1640-1660.
- Ait-Oufella, H., Salomon, B.L., Potteaux, S., Robertson, A.K., Gourdy, P., Zoll, J., Merval, R., Esposito, B., Cohen, J.L., Fisson, S., *et al.* (2006). Natural regulatory T cells control the development of atherosclerosis in mice. *Nature medicine* 12, 178-180.
- Ait-Oufella, H., Taleb, S., Mallat, Z., and Tedgui, A. (2011). Recent Advances on the Role of Cytokines in Atherosclerosis. *Arteriosclerosis, Thrombosis, and Vascular Biology* 31, 969-979.
- Ayrolidi, E., Cannarile, L., Migliorati, G., Nocentini, G., Delfino, D.V., and Riccardi, C. (2012). Mechanisms of the anti-inflammatory effects of glucocorticoids: genomic and nongenomic interference with MAPK signaling pathways. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 26, 4805-4820.
- Backteman, K., Ernerudh, J., and Jonasson, L. (2014). Natural killer (NK) cell deficit in coronary artery disease: no aberrations in phenotype but sustained reduction of NK cells is associated with low-grade inflammation. *Clinical and experimental immunology* 175, 104-112.
- Bagnato, C., Thumar, J., Mayya, V., Hwang, S.I., Zebroski, H., Claffey, K.P., Haudenschild, C., Eng, J.K., Lundgren, D.H., and Han, D.K. (2007). Proteomics analysis of human coronary atherosclerotic plaque: a feasibility study of direct tissue proteomics by liquid chromatography and tandem mass spectrometry. *Molecular & cellular proteomics : MCP* 6, 1088-1102.

- Berg, K.E., Ljungcrantz, I., Andersson, L., Bryngelsson, C., Hedblad, B., Fredrikson, G.N., Nilsson, J., and Bjorkbacka, H. (2012). Elevated CD14++CD16- monocytes predict cardiovascular events. *Circulation. Cardiovascular genetics* 5, 122-131.
- Buckingham, J.C. (2006). Glucocorticoids: exemplars of multi-tasking. *British journal of pharmacology* 147 Suppl 1, S258-268.
- Buffon, A., Biasucci, L.M., Liuzzo, G., D'Onofrio, G., Crea, F., and Maseri, A. (2002). Widespread coronary inflammation in unstable angina. *The New England journal of medicine* 347, 5-12.
- Burgmaier, M., Schutters, K., Willems, B., van der Vorst, E.P., Kusters, D., Chatrou, M., Norling, L., Biessen, E.A., Cleutjens, J., Perretti, M., *et al.* (2014). AnxA5 reduces plaque inflammation of advanced atherosclerotic lesions in apoE(-/-) mice. *Journal of cellular and molecular medicine* 18, 2117-2124.
- Busillo, J.M., and Cidlowski, J.A. (2013). The five Rs of glucocorticoid action during inflammation: ready, reinforce, repress, resolve, and restore. *Trends in endocrinology and metabolism: TEM* 24, 109-119.
- Caidahl, K., Hartford, M., Karlsson, T., Herlitz, J., Pettersson, K., de Faire, U., and Frostegard, J. (2013). IgM-phosphorylcholine autoantibodies and outcome in acute coronary syndromes. *International journal of cardiology* 167, 464-469.
- Caligiuri, G., Liuzzo, G., Biasucci, L.M., and Maseri, A. (1998). Immune system activation follows inflammation in unstable angina: pathogenetic implications. *Journal of the American College of Cardiology* 32, 1295-1304.
- Calvert, P.A., Liew, T.V., Gorenne, I., Clarke, M., Costopoulos, C., Obaid, D.R., O'Sullivan, M., Shapiro, L.M., McNab, D.C., Densem, C.G., *et al.* (2011). Leukocyte telomere length is associated with high-risk plaques on virtual histology intravascular ultrasound and increased proinflammatory activity. *Arterioscler Thromb Vasc Biol* 31, 2157-2164.
- Campbell, K.S., and Hasegawa, J. (2013). Natural killer cell biology: an update and future directions. *The Journal of allergy and clinical immunology* 132, 536-544.
- Campeau, L. (1976). Letter: Grading of angina pectoris. *Circulation* 54, 522-523.
- Cheng, X., Yu, X., Ding, Y.J., Fu, Q.Q., Xie, J.J., Tang, T.T., Yao, R., Chen, Y., and Liao, Y.H. (2008). The Th17/Treg imbalance in patients with acute coronary syndrome. *Clin Immunol* 127, 89-97.
- Cheuk, B.L., and Cheng, S.W. (2011). Annexin A1 expression in atherosclerotic carotid plaques and its relationship with plaque characteristics. *European journal of vascular*

and endovascular surgery : the official journal of the European Society for Vascular Surgery 41, 364-371.

Comera, C., and Russo-Marie, F. (1995). Glucocorticoid-induced annexin 1 secretion by monocytes and peritoneal leukocytes. *British journal of pharmacology* 115, 1043-1047.

Correia, M.P., Costa, A.V., Uhrberg, M., Cardoso, E.M., and Arosa, F.A. (2011). IL-15 induces CD8+ T cells to acquire functional NK receptors capable of modulating cytotoxicity and cytokine secretion. *Immunobiology* 216, 604-612.

D'Acquisto, F., Merghani, A., Lecona, E., Rosignoli, G., Raza, K., Buckley, C.D., Flower, R.J., and Perretti, M. (2007a). Annexin-1 modulates T-cell activation and differentiation. *Blood* 109, 1095-1102.

D'Acquisto, F., Paschalidis, N., Raza, K., Buckley, C.D., Flower, R.J., and Perretti, M. (2008a). Glucocorticoid treatment inhibits annexin-1 expression in rheumatoid arthritis CD4+ T cells. *Rheumatology* 47, 636-639.

D'Acquisto, F., Paschalidis, N., Sampaio, A.L., Merghani, A., Flower, R.J., and Perretti, M. (2007b). Impaired T cell activation and increased Th2 lineage commitment in Annexin-1-deficient T cells. *European journal of immunology* 37, 3131-3142.

D'Acquisto, F., Perretti, M., and Flower, R.J. (2008b). Annexin-A1: a pivotal regulator of the innate and adaptive immune systems. *British journal of pharmacology* 155, 152-169.

D'Acunto, C.W., Gbelcova, H., Festa, M., and Ruml, T. (2014). The complex understanding of Annexin A1 phosphorylation. *Cellular signalling* 26, 173-178.

Dawber, T.R., Kannel, W.B., Revotskie, N., Stokes, J., 3rd, Kagan, A., and Gordon, T. (1959). Some factors associated with the development of coronary heart disease: six years' follow-up experience in the Framingham study. *American journal of public health and the nation's health* 49, 1349-1356.

Dawber, T.R., Moore, F.E., and Mann, G.V. (1957). Coronary heart disease in the Framingham study. *American journal of public health and the nation's health* 47, 4-24.

Davila, E., Kang, Y.M., Park, Y.W., Sawai, H., He, X., Pryshchep, S., Goronzy, J.J., and Weyand, C.M. (2005). Cell-Based Immunotherapy with Suppressor CD8+ T Cells in Rheumatoid Arthritis. *The Journal of Immunology* 174, 7292-7301.

de Waal Malefyt, R., Abrams, J., Bennett, B., Figdor, C.G., and de Vries, J.E. (1991). Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an

autoregulatory role of IL-10 produced by monocytes. *The Journal of experimental medicine* 174, 1209-1220.

Dekker, M.J., Koper, J.W., van Aken, M.O., Pols, H.A., Hofman, A., de Jong, F.H., Kirschbaum, C., Witteman, J.C., Lamberts, S.W., and Tiemeier, H. (2008). Salivary cortisol is related to atherosclerosis of carotid arteries. *The Journal of clinical endocrinology and metabolism* 93, 3741-3747.

Doring, Y., Drechsler, M., Soehnlein, O., and Weber, C. (2014). Neutrophils in Atherosclerosis: From Mice to Man. *Arterioscler Thromb Vasc Biol*.

Drechsler, M., de Jong, R.J., Rossaint, J., Viola, J., Leoni, G., Wang, J.M., Grommes, J., Hinkel, R., Kupatt, C., Weber, C., *et al.* (2014). Annexin A1 Counteracts Chemokine-Induced Arterial Myeloid Cell Recruitment. *Circ Res*.

Egger, G., Burda, A., Obernosterer, A., Mitterhammer, H., Kager, G., Jurgens, G., Hofer, H.P., Fabjan, J.S., and Pilger, E. (2001). Blood polymorphonuclear leukocyte activation in atherosclerosis: effects of aspirin. *Inflammation* 25, 129-135.

Elsenberg, E.H., Sels, J.E., Hillaert, M.A., Schoneveld, A.H., van den Dungen, N.A., van Holten, T.C., Roest, M., Jukema, J.W., van Zonneveld, A.J., de Groot, P.G., *et al.* (2013). Increased cytokine response after toll-like receptor stimulation in patients with stable coronary artery disease. *Atherosclerosis* 231, 346-351.

Fahey, A.J., Robins, R.A., Kindle, K.B., Heery, D.M., and Constantinescu, C.S. (2006). Effects of glucocorticoids on STAT4 activation in human T cells are stimulus-dependent. *Journal of leukocyte biology* 80, 133-144.

Fantidis, P., Perez De Prada, T., Fernandez-Ortiz, A., Carcia-Touchard, A., Alfonso, F., Sabate, M., Hernandez, R., Escaned, J., Baelos, C., and Macaya, C. (2002). Morning cortisol production in coronary heart disease patients. *European journal of clinical investigation* 32, 304-308.

Ferlazzo, V., D'Agostino, P., Milano, S., Caruso, R., Feo, S., Cillari, E., and Parente, L. (2003). Anti-inflammatory effects of annexin-1: stimulation of IL-10 release and inhibition of nitric oxide synthesis. *International immunopharmacology* 3, 1363-1369.

Franchimont, D., Galon, J., Gadina, M., Visconti, R., Zhou, Y., Aringer, M., Frucht, D.M., Chrousos, G.P., and O'Shea, J.J. (2000). Inhibition of Th1 immune response by glucocorticoids: dexamethasone selectively inhibits IL-12-induced Stat4 phosphorylation in T lymphocytes. *Journal of immunology* 164, 1768-1774.

- Friedman, G.D., Klatsky, A.L., and Siegelaub, A.B. (1974). The leukocyte count as a predictor of myocardial infarction. *The New England journal of medicine* 290, 1275-1278.
- Frostegard, J. (2013). Immunity, atherosclerosis and cardiovascular disease. *BMC medicine* 11, 117.
- Frostegard, J., Ulfgren, A.K., Nyberg, P., Hedin, U., Swedenborg, J., Andersson, U., and Hansson, G.K. (1999). Cytokine expression in advanced human atherosclerotic plaques: dominance of pro-inflammatory (Th1) and macrophage-stimulating cytokines. *Atherosclerosis* 145, 33-43.
- Gerke, V., and Moss, S.E. (2002). Annexins: from structure to function. *Physiological reviews* 82, 331-371.
- Gewaltig, J., Kummer, M., Koella, C., Cathomas, G., and Biedermann, B.C. (2008). Requirements for CD8 T-cell migration into the human arterial wall. *Human Pathology* 39, 1756-1762.
- Gigante, B., Leander, K., Vikstrom, M., Baldassarre, D., Veglia, F., Strawbridge, R.J., McLeod, O., Gertow, K., Sennblad, B., Shah, S., *et al.* (2014). Low levels of IgM antibodies against phosphorylcholine are associated with fast carotid intima media thickness progression and cardiovascular risk in men. *Atherosclerosis* 236, 394-399.
- Ginhoux, F., and Jung, S. (2014). Monocytes and macrophages: developmental pathways and tissue homeostasis. *Nature reviews. Immunology* 14, 392-404.
- Gold, R., Pepinsky, R.B., Zettl, U.K., Toyka, K.V., and Hartung, H.P. (1996). Lipocortin-1 (annexin-1) suppresses activation of autoimmune T cell lines in the Lewis rat. *Journal of neuroimmunology* 69, 157-164.
- Goldsby, R.A., and Goldsby, R.A. (2003). *Immunology*, 5th edn (New York: W.H. Freeman).
- Goulding, N.J., Godolphin, J.L., Sharland, P.R., Peers, S.H., Sampson, M., Maddison, P.J., and Flower, R.J. (1990). Anti-inflammatory lipocortin 1 production by peripheral blood leucocytes in response to hydrocortisone. *Lancet* 335, 1416-1418.
- Grivel, J.-C., Ivanova, O., Pinegina, N., Blank, P.S., Shpektor, A., Margolis, L.B., and Vasilieva, E. (2011). Activation of T Lymphocytes in Atherosclerotic Plaques. *Arteriosclerosis, Thrombosis, and Vascular Biology* 31, 2929-2937.
- Guasti, L., Dentali, F., Castiglioni, L., Maroni, L., Marino, F., Squizzato, A., Ageno, W., Gianni, M., Gaudio, G., Grandi, A.M., *et al.* (2011). Neutrophils and clinical outcomes in patients with acute coronary syndromes and/or cardiac revascularisation. *A*

systematic review on more than 34,000 subjects. *Thrombosis and haemostasis* 106, 591-599.

Guasti, L., Marino, F., Cosentino, M., Cimpanelli, M., Maio, R.C., Klersy, C., Crespi, C., Restelli, D., Simoni, C., Franzetti, I., *et al.* (2006). Simvastatin treatment modifies polymorphonuclear leukocyte function in high-risk individuals: a longitudinal study. *J Hypertens* 24, 2423-2430.

Hagendorf, A., Koper, J.W., de Jong, F.H., Brinkmann, A.O., Lamberts, S.W., and Feelders, R.A. (2005). Expression of the human glucocorticoid receptor splice variants alpha, beta, and P in peripheral blood mononuclear leukocytes in healthy controls and in patients with hyper- and hypocortisolism. *The Journal of clinical endocrinology and metabolism* 90, 6237-6243.

Hak, L., Mysliwska, J., Wieckiewicz, J., Szyndler, K., Trzonkowski, P., Siebert, J., and Mysliwski, A. (2007). NK cell compartment in patients with coronary heart disease. *Immunity & ageing : I & A* 4, 3.

Han, S.F., Liu, P., Zhang, W., Bu, L., Shen, M., Li, H., Fan, Y.H., Cheng, K., Cheng, H.X., Li, C.X., and Jia, G.L. (2007). The opposite-direction modulation of CD4+CD25+ Tregs and T helper 1 cells in acute coronary syndromes. *Clin Immunol* 124, 90-97.

Hansson, G.K. (2005). Inflammation, atherosclerosis, and coronary artery disease. *The New England journal of medicine* 352, 1685-1695.

Hansson, G.K., and Hermansson, A. (2011). The immune system in atherosclerosis. *Nat Immunol* 12, 204-212.

Hansson, G.K., and Libby, P. (2006). The immune response in atherosclerosis: a double-edged sword. *Nature reviews. Immunology* 6, 508-519.

Hartwig, H., Silvestre Roig, C., Daemen, M., Lutgens, E., and Soehnlein, O. (2014). Neutrophils in atherosclerosis. A brief overview. *Hamostaseologie* 35.

Hayes, M.J., and Moss, S.E. (2004). Annexins and disease. *Biochemical and biophysical research communications* 322, 1166-1170.

Hellhammer, J., Fries, E., Schweisthal, O.W., Schlotz, W., Stone, A.A., and Hagemann, D. (2007). Several daily measurements are necessary to reliably assess the cortisol rise after awakening: state- and trait components. *Psychoneuroendocrinology* 32, 80-86.

Hilgendorf, I., Swirski, F.K., and Robbins, C.S. (2014). Monocyte Fate in Atherosclerosis. *Arterioscler Thromb Vasc Biol*.

- Ionita, M.G., van den Borne, P., Catanzariti, L.M., Moll, F.L., de Vries, J.P., Pasterkamp, G., Vink, A., and de Kleijn, D.P. (2010). High neutrophil numbers in human carotid atherosclerotic plaques are associated with characteristics of rupture-prone lesions. *Arterioscler Thromb Vasc Biol* 30, 1842-1848.
- Jaillon, S., Galdiero, M.R., Del Prete, D., Cassatella, M.A., Garlanda, C., and Mantovani, A. (2013). Neutrophils in innate and adaptive immunity. *Seminars in immunopathology* 35, 377-394.
- Janeway, C. (2005). *Immunobiology : the immune system in health and disease*, 6th edn (New York: Garland Science).
- Jonasson, L., Backteman, K., and Ernerudh, J. (2005). Loss of natural killer cell activity in patients with coronary artery disease. *Atherosclerosis* 183, 316-321.
- Jonasson, L., Holm, J., Skalli, O., Bondjers, G., and Hansson, G.K. (1986). Regional accumulations of T cells, macrophages, and smooth muscle cells in the human atherosclerotic plaque. *Arteriosclerosis* 6, 131-138.
- Jonasson, L., Tompa, A., and Wikby, A. (2003). Expansion of peripheral CD8+ T cells in patients with coronary artery disease: relation to cytomegalovirus infection. *Journal of Internal Medicine* 254, 472-478.
- Jonsson, S., Lundberg, A., Kalvegren, H., Bergstrom, I., Szymanowski, A., and Jonasson, L. (2011). Increased levels of leukocyte-derived MMP-9 in patients with stable angina pectoris. *PLoS One* 6, e19340.
- Kadmiel, M., and Cidowski, J.A. (2013). Glucocorticoid receptor signaling in health and disease. *Trends in pharmacological sciences* 34, 518-530.
- Kamal, A.M., Smith, S.F., De Silva Wijayasinghe, M., Solito, E., and Corrigan, C.J. (2001). An annexin 1 (ANXA1)-derived peptide inhibits prototype antigen-driven human T cell Th1 and Th2 responses in vitro. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 31, 1116-1125.
- Kannel, W.B., Dawber, T.R., Kagan, A., Revotskie, N., and Stokes, J., 3rd (1961). Factors of risk in the development of coronary heart disease--six year follow-up experience. The Framingham Study. *Annals of internal medicine* 55, 33-50.
- Katchar, K., Söderström, K., Wahlstrom, J., Eklund, A., and Grunewald, J. (2005). Characterisation of natural killer cells and CD56+ T-cells in sarcoidosis patients. *European Respiratory Journal* 26, 77-85.
- Kelly-Rogers, J., Madrigal-Estebas, L., O'Connor, T., and Doherty, D.G. (2006). Activation-Induced Expression of CD56 by T Cells Is Associated With a

- Reprogramming of Cytolytic Activity and Cytokine Secretion Profile In Vitro. *Human Immunology* 67, 863-873.
- Kirschbaum, C., and Hellhammer, D.H. (1999). Noise and Stress - Salivary Cortisol as a Non-Invasive Measure of Allostatic Load. *Noise & health* 1, 57-66.
- Klinker, M.W., and Lundy, S.K. (2012). Multiple mechanisms of immune suppression by B lymphocytes. *Molecular medicine* 18, 123-137.
- Koch, S., Larbi, A., Ozcelik, D., Solana, R., Gouttefangeas, C., Attig, S., Wikby, A., Strindhall, J., Franceschi, C., and Pawelec, G. (2007). Cytomegalovirus infection: a driving force in human T cell immunosenescence. *Ann N Y Acad Sci* 1114, 23-35.
- Koertge, J., Al-Khalili, F., Ahnve, S., Janszky, I., Svane, B., and Schenck-Gustafsson, K. (2002). Cortisol and vital exhaustion in relation to significant coronary artery stenosis in middle-aged women with acute coronary syndrome. *Psychoneuroendocrinology* 27, 893-906.
- Kolbus, D., Ramos, O.H., Berg, K.E., Persson, J., Wigren, M., Bjorkbacka, H., Fredrikson, G.N., and Nilsson, J. (2010). CD8+ T cell activation predominate early immune responses to hypercholesterolemia in Apoe(-)/(-) mice. *BMC immunology* 11, 58.
- Kostis, J.B., Turkevich, D., and Sharp, J. (1984). Association between leukocyte count and the presence and extent of coronary atherosclerosis as determined by coronary arteriography. *Am J Cardiol* 53, 997-999.
- Lange, C., Starrett, D.J., Goetsch, J., Gerke, V., and Rescher, U. (2007). Transcriptional profiling of human monocytes reveals complex changes in the expression pattern of inflammation-related genes in response to the annexin A1-derived peptide Ac1-25. *Journal of leukocyte biology* 82, 1592-1604.
- Lemster, B.H., Michel, J.J., Montag, D.T., Paat, J.J., Studenski, S.A., Newman, A.B., and Vallejo, A.N. (2008). Induction of CD56 and TCR-independent activation of T cells with aging. *Journal of immunology* 180, 1979-1990.
- Li, X., Zhang, F.S., Zhang, J.H., and Wang, J.Y. (2010). Negative relationship between expression of glucocorticoid receptor alpha and disease activity: glucocorticoid treatment of patients with systemic lupus erythematosus. *The Journal of rheumatology* 37, 316-321.
- Libby, P. (2012). Inflammation in atherosclerosis. *Arterioscler Thromb Vasc Biol* 32, 2045-2051.

- Lindmark, E., Wallentin, L., and Siegbahn, A. (2001). Blood cell activation, coagulation, and inflammation in men and women with coronary artery disease. *Thrombosis research* 103, 249-259.
- Liu, A., Ming, J.Y., Fiskesund, R., Ninio, E., Karabina, S.A., Bergmark, C., Frostegard, A.G., and Frostegard, J. (2015). Induction of dendritic cell-mediated T-cell activation by modified but not native low-density lipoprotein in humans and inhibition by annexin a5: involvement of heat shock proteins. *Arterioscler Thromb Vasc Biol* 35, 197-205.
- Malarstig, A., Eriksson, P., Hamsten, A., Lindahl, B., Wallentin, L., and Siegbahn, A. (2008). Raised interleukin-10 is an indicator of poor outcome and enhanced systemic inflammation in patients with acute coronary syndrome. *Heart* 94, 724-729.
- Matthews, K., Schwartz, J., Cohen, S., and Seeman, T. (2006). Diurnal cortisol decline is related to coronary calcification: CARDIA study. *Psychosomatic medicine* 68, 657-661.
- McLeod, J.D., and Bolton, C. (1995). Dexamethasone induces an increase in intracellular and membrane-associated lipocortin-1 (annexin-1) in rat astrocyte primary cultures. *Cellular and molecular neurobiology* 15, 193-205.
- McLeod, J.D., Goodall, A., Jelic, P., and Bolton, C. (1995). Changes in the cellular distribution of lipocortin-1 (Annexin-1) in C6 glioma cells after exposure to dexamethasone. *Biochemical pharmacology* 50, 1103-1107.
- Mehta, J., Dinerman, J., Mehta, P., Saldeen, T.G., Lawson, D., Donnelly, W.H., and Wallin, R. (1989). Neutrophil function in ischemic heart disease. *Circulation* 79, 549-556.
- Methe, H., Brunner, S., Wiegand, D., Nabauer, M., Koglin, J., and Edelman, E.R. (2005). Enhanced T-Helper-1 Lymphocyte Activation Patterns in Acute Coronary Syndromes. *Journal of the American College of Cardiology* 45, 1939-1945.
- Michel, J.J., Turesson, C., Lemster, B., Atkins, S.R., Iclozan, C., Bongartz, T., Wasko, M.C., Matteson, E.L., and Vallejo, A.N. (2007). CD56-expressing T cells that have features of senescence are expanded in rheumatoid arthritis. *Arthritis & Rheumatism* 56, 43-57.
- Miller, Y.I., Choi, S.H., Wiesner, P., Fang, L., Harkewicz, R., Hartvigsen, K., Boullier, A., Gonen, A., Diehl, C.J., Que, X., *et al.* (2011). Oxidation-specific epitopes are danger-associated molecular patterns recognized by pattern recognition receptors of innate immunity. *Circ Res* 108, 235-248.

- Moore, K.J., Sheedy, F.J., and Fisher, E.A. (2013). Macrophages in atherosclerosis: a dynamic balance. *Nature reviews. Immunology* 13, 709-721.
- Mor, A., Luboshits, G., Planer, D., Keren, G., and George, J. (2006). Altered status of CD4(+)CD25(+) regulatory T cells in patients with acute coronary syndromes. *European heart journal* 27, 2530-2537.
- Mor, A., Planer, D., Luboshits, G., Afek, A., Metzger, S., Chajek-Shaul, T., Keren, G., and George, J. (2007). Role of naturally occurring CD4⁺ CD25⁺ regulatory T cells in experimental atherosclerosis. *Arterioscler Thromb Vasc Biol* 27, 893-900.
- Morand, E.F., Hutchinson, P., Hargreaves, A., Goulding, N.J., Boyce, N.W., and Holdsworth, S.R. (1995). Detection of intracellular lipocortin 1 in human leukocyte subsets. *Clinical immunology and immunopathology* 76, 195-202.
- Morand, E.F., Jefferiss, C.M., Dixey, J., Mitra, D., and Goulding, N.J. (1994). Impaired glucocorticoid induction of mononuclear leukocyte lipocortin-1 in rheumatoid arthritis. *Arthritis and rheumatism* 37, 207-211.
- Moretta, L., Montaldo, E., Vacca, P., Del Zotto, G., Moretta, F., Merli, P., Locatelli, F., and Mingari, M.C. (2014). Human natural killer cells: origin, receptors, function, and clinical applications. *International archives of allergy and immunology* 164, 253-264.
- Mortality, G.B.D., and Causes of Death, C. (2014). Global, regional, and national age-sex specific all-cause and cause-specific mortality for 240 causes of death, 1990-2013: a systematic analysis for the Global Burden of Disease Study 2013. *Lancet*.
- Moss, S.E., and Morgan, R.O. (2004). The annexins. *Genome biology* 5, 219.
- Mulla, A., Leroux, C., Solito, E., and Buckingham, J.C. (2005). Correlation between the antiinflammatory protein annexin 1 (lipocortin 1) and serum cortisol in subjects with normal and dysregulated adrenal function. *The Journal of clinical endocrinology and metabolism* 90, 557-562.
- Murray, C.J., Vos, T., Lozano, R., Naghavi, M., Flaxman, A.D., Michaud, C., Ezzati, M., Shibuya, K., Salomon, J.A., Abdalla, S., *et al.* (2012). Disability-adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet* 380, 2197-2223.
- Naruko, T., Ueda, M., Haze, K., van der Wal, A.C., van der Loos, C.M., Itoh, A., Komatsu, R., Ikura, Y., Ogami, M., Shimada, Y., *et al.* (2002). Neutrophil infiltration of culprit lesions in acute coronary syndromes. *Circulation* 106, 2894-2900.

- Neri Serneri, G.G., Prisco, D., Martini, F., Gori, A.M., Brunelli, T., Poggesi, L., Rostagno, C., Gensini, G.F., and Abbate, R. (1997). Acute T-cell activation is detectable in unstable angina. *Circulation* 95, 1806-1812.
- Nijm, J., Kristenson, M., Olsson, A.G., and Jonasson, L. (2007). Impaired cortisol response to acute stressors in patients with coronary disease. Implications for inflammatory activity. *J Intern Med* 262, 375-384.
- Nijm, J., Wikby, A., Tompa, A., Olsson, A.G., and Jonasson, L. (2005). Circulating levels of proinflammatory cytokines and neutrophil-platelet aggregates in patients with coronary artery disease. *Am J Cardiol* 95, 452-456.
- Oakley, R.H., and Cidlowski, J.A. (2013). The biology of the glucocorticoid receptor: new signaling mechanisms in health and disease. *The Journal of allergy and clinical immunology* 132, 1033-1044.
- Ohkawa, T., Seki, S., Dobashi, H., Koike, Y., Habu, Y., Ami, K., Hiraide, H., and Sekine, I. (2001). Systematic characterization of human CD8+ T cells with natural killer cell markers in comparison with natural killer cells and normal CD8+ T cells. *Immunology* 103, 281-290.
- Ortega-Gomez, A., Perretti, M., and Soehnlein, O. (2013). Resolution of inflammation: an integrated view. *EMBO molecular medicine* 5, 661-674.
- Ott, I., Neumann, F.J., Gawaz, M., Schmitt, M., and Schomig, A. (1996). Increased neutrophil-platelet adhesion in patients with unstable angina. *Circulation* 94, 1239-1246.
- Packard, R.R., Lichtman, A.H., and Libby, P. (2009). Innate and adaptive immunity in atherosclerosis. *Seminars in immunopathology* 31, 5-22.
- Panda, S., and Ding, J.L. (2015). Natural Antibodies Bridge Innate and Adaptive Immunity. *Journal of immunology* 194, 13-20.
- Paschalidis, N., Iqbal, A.J., Maione, F., Wood, E.G., Perretti, M., Flower, R.J., and D'Acquisto, F. (2009). Modulation of experimental autoimmune encephalomyelitis by endogenous annexin A1. *Journal of neuroinflammation* 6, 33.
- Patel, K.D., Duggan, S.P., Currid, C.A., Gallagher, W.M., McManus, R., Kelleher, D., Murphy, R.T., and Ryan, A.W. (2009). High sensitivity cytokine detection in acute coronary syndrome reveals up-regulation of Interferon Gamma and Interleukin-10 post Myocardial Infarction. *Clinical Immunology* 133, 251-256.

- Paulsson, J., Dadfar, E., Held, C., Jacobson, S.H., and Lundahl, J. (2007). Activation of peripheral and in vivo transmigrated neutrophils in patients with stable coronary artery disease. *Atherosclerosis* 192, 328-334.
- Peralbo, E., Alonso, C., and Solana, R. (2007). Invariant NKT and NKT-like lymphocytes: two different T cell subsets that are differentially affected by ageing. *Exp Gerontol* 42, 703-708.
- Perretti, M., and D'Acquisto, F. (2009). Annexin A1 and glucocorticoids as effectors of the resolution of inflammation. *Nature reviews. Immunology* 9, 62-70.
- Perretti, M., and Flower, R.J. (2004). Annexin 1 and the biology of the neutrophil. *Journal of leukocyte biology* 76, 25-29.
- Perretti, M., and Solito, E. (2004). Annexin 1 and neutrophil apoptosis. *Biochemical Society transactions* 32, 507-510.
- Perry, H.M., Bender, T.P., and McNamara, C.A. (2012). B cell subsets in atherosclerosis. *Frontiers in immunology* 3, 373.
- Pimenta, E., Wolley, M., and Stowasser, M. (2012). Adverse cardiovascular outcomes of corticosteroid excess. *Endocrinology* 153, 5137-5142.
- Pittet, M.J., Speiser, D.E., Valmori, D., Cerottini, J.C., and Romero, P. (2000). Cutting edge: cytolytic effector function in human circulating CD8+ T cells closely correlates with CD56 surface expression. *Journal of immunology* 164, 1148-1152.
- Pruessner, J.C., Wolf, O.T., Hellhammer, D.H., Buske-Kirschbaum, A., von Auer, K., Jobst, S., Kaspers, F., and Kirschbaum, C. (1997). Free cortisol levels after awakening: a reliable biological marker for the assessment of adrenocortical activity. *Life sciences* 61, 2539-2549.
- Qiao, J.H., and Fishbein, M.C. (1991). The severity of coronary atherosclerosis at sites of plaque rupture with occlusive thrombosis. *J Am Coll Cardiol* 17, 1138-1142.
- Quax, R.A., Manenschijn, L., Koper, J.W., Hazes, J.M., Lamberts, S.W., van Rossum, E.F., and Feelders, R.A. (2013). Glucocorticoid sensitivity in health and disease. *Nature reviews. Endocrinology* 9, 670-686.
- Rang, H.P., Dale, M.M., Ritter, J.M., and Flower, R.J. (2007). *Rang and Dale's Pharmacology*, 6th edn (Philadelphia: Churchill Livingstone Elsevier).
- Rogacev, K.S., Cremers, B., Zawada, A.M., Seiler, S., Binder, N., Ege, P., Grosse-Dunker, G., Heisel, I., Hornof, F., Jeken, J., *et al.* (2012). CD14++CD16+ monocytes

- independently predict cardiovascular events: a cohort study of 951 patients referred for elective coronary angiography. *J Am Coll Cardiol* 60, 1512-1520.
- Romo, N., Fitó, M., Gumá, M., Sala, J., García, C., Ramos, R., Muntasell, A., Masiá, R., Bruguera, J., Subirana, I., *et al.* (2011). Association of Atherosclerosis With Expression of the LILRB1 Receptor By Human NK and T-Cells Supports the Infectious Burden Hypothesis. *Arteriosclerosis, Thrombosis, and Vascular Biology* 31, 2314-2321.
- Rosengarth, A., and Luecke, H. (2003). A calcium-driven conformational switch of the N-terminal and core domains of annexin A1. *Journal of molecular biology* 326, 1317-1325.
- Rosengren, A., Hawken, S., Ounpuu, S., Sliwa, K., Zubaid, M., Almahmeed, W.A., Blackett, K.N., Sittithi-amorn, C., Sato, H., Yusuf, S., and investigators, I. (2004). Association of psychosocial risk factors with risk of acute myocardial infarction in 11119 cases and 13648 controls from 52 countries (the INTERHEART study): case-control study. *Lancet* 364, 953-962.
- Samarasinghe, R.A., Witchell, S.F., and DeFranco, D.B. (2012). Cooperativity and complementarity: synergies in non-classical and classical glucocorticoid signaling. *Cell cycle* 11, 2819-2827.
- Sarndahl, E., Bergstrom, I., Brodin, V.P., Nijm, J., Lundqvist Setterud, H., and Jonasson, L. (2007). Neutrophil activation status in stable coronary artery disease. *PLoS One* 2, e1056.
- Schlaghecke, R., Kornely, E., Wollenhaupt, J., and Specker, C. (1992). Glucocorticoid receptors in rheumatoid arthritis. *Arthritis and rheumatism* 35, 740-744.
- Serhan, C.N. (2014). Pro-resolving lipid mediators are leads for resolution physiology. *Nature* 510, 92-101.
- Serhan, C.N., Brain, S.D., Buckley, C.D., Gilroy, D.W., Haslett, C., O'Neill, L.A., Perretti, M., Rossi, A.G., and Wallace, J.L. (2007). Resolution of inflammation: state of the art, definitions and terms. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 21, 325-332.
- Serhan, C.N., Ward, P.A., and Gilroy, D.W. (2010). *Fundamentals of inflammation* (Cambridge ; New York: Cambridge University Press).
- Shimamoto, M., Ueno, Y., Tanaka, S., Onitake, T., Hanaoka, R., Yoshioka, K., Hatakeyama, T., and Chayama, K. (2007). Selective decrease in colonic CD56(+) T and CD161(+) T cells in the inflamed mucosa of patients with ulcerative colitis. *World journal of gastroenterology : WJG* 13, 5995-6002.

- Silverman, M.N., and Sternberg, E.M. (2012). Glucocorticoid regulation of inflammation and its functional correlates: from HPA axis to glucocorticoid receptor dysfunction. *Ann N Y Acad Sci* 1261, 55-63.
- Simanek, A.M., Dowd, J.B., Pawelec, G., Melzer, D., Dutta, A., and Aiello, A.E. (2011). Seropositivity to Cytomegalovirus, Inflammation, All-Cause and Cardiovascular Disease-Related Mortality in the United States. *PLoS ONE* 6, e16103.
- Soehnlein, O. (2012). Multiple roles for neutrophils in atherosclerosis. *Circ Res* 110, 875-888.
- Solito, E., Mulla, A., Morris, J.F., Christian, H.C., Flower, R.J., and Buckingham, J.C. (2003). Dexamethasone induces rapid serine-phosphorylation and membrane translocation of annexin 1 in a human folliculostellate cell line via a novel nongenomic mechanism involving the glucocorticoid receptor, protein kinase C, phosphatidylinositol 3-kinase, and mitogen-activated protein kinase. *Endocrinology* 144, 1164-1174.
- Spurr, L., Nadkarni, S., Pederzoli-Ribeil, M., Goulding, N.J., Perretti, M., and D'Acquisto, F. (2011). Comparative analysis of Annexin A1-formyl peptide receptor 2/ALX expression in human leukocyte subsets. *International immunopharmacology* 11, 55-66.
- Strausbaugh, H.J., and Rosen, S.D. (2001). A potential role for annexin 1 as a physiologic mediator of glucocorticoid-induced L-selectin shedding from myeloid cells. *Journal of immunology* 166, 6294-6300.
- Sun, J.C., and Lanier, L.L. (2011). NK cell development, homeostasis and function: parallels with CD8(+) T cells. *Nature reviews. Immunology* 11, 645-657.
- Tabas, I., Williams, K.J., and Boren, J. (2007). Subendothelial lipoprotein retention as the initiating process in atherosclerosis: update and therapeutic implications. *Circulation* 116, 1832-1844.
- Tacke, F., and Randolph, G.J. (2006). Migratory fate and differentiation of blood monocyte subsets. *Immunobiology* 211, 609-618.
- Tarazona, R., DelaRosa, O., Alonso, C., Ostos, B., Espejo, J., Pena, J., and Solana, R. (2000). Increased expression of NK cell markers on T lymphocytes in aging and chronic activation of the immune system reflects the accumulation of effector/senescent T cells. *Mechanisms of ageing and development* 121, 77-88.
- Thygesen, K., Alpert, J.S., Jaffe, A.S., Simoons, M.L., Chaitman, B.R., White, H.D., Joint, E.S.C.A.A.H.A.W.H.F.T.F.f.U.D.o.M.I., Authors/Task Force Members, C., Thygesen,

- K., Alpert, J.S., *et al.* (2012). Third universal definition of myocardial infarction. *J Am Coll Cardiol* 60, 1581-1598.
- Tsiantoulas, D., Diehl, C.J., Witztum, J.L., and Binder, C.J. (2014a). B cells and humoral immunity in atherosclerosis. *Circ Res* 114, 1743-1756.
- Tsiantoulas, D., Sage, A.P., Mallat, Z., and Binder, C.J. (2014b). Targeting B Cells in Atherosclerosis: Closing the Gap From Bench to Bedside. *Arterioscler Thromb Vasc Biol*.
- Waehre, T., Halvorsen, B., Damas, J.K., Yndestad, A., Brosstad, F., Gullestad, L., Kjekshus, J., Froland, S.S., and Aukrust, P. (2002). Inflammatory imbalance between IL-10 and TNF α in unstable angina potential plaque stabilizing effects of IL-10. *European journal of clinical investigation* 32, 803-810.
- Waehre, T., Yndestad, A., Smith, C., Haug, T., Tunheim, S.H., Gullestad, L., Froland, S.S., Semb, A.G., Aukrust, P., and Damas, J.K. (2004). Increased expression of interleukin-1 in coronary artery disease with downregulatory effects of HMG-CoA reductase inhibitors. *Circulation* 109, 1966-1972.
- van Erk, M.J., Wopereis, S., Rubingh, C., van Vliet, T., Verheij, E., Cnubben, N.H., Pedersen, T.L., Newman, J.W., Smilde, A.K., van der Greef, J., *et al.* (2010). Insight in modulation of inflammation in response to diclofenac intervention: a human intervention study. *BMC medical genomics* 3, 5.
- Wan, Y.Y. (2010). Multi-tasking of helper T cells. *Immunology* 130, 166-171.
- Wang, J., and Arase, H. (2014). Regulation of immune responses by neutrophils. *Ann N Y Acad Sci* 1319, 66-81.
- Wang, J., Guo, C., Liu, S., Qi, H., Yin, Y., Liang, R., Sun, M.Z., and Greenaway, F.T. (2014). Annexin A11 in disease. *Clinica chimica acta; international journal of clinical chemistry* 431, 164-168.
- Weber, C., and Noels, H. (2011). Atherosclerosis: current pathogenesis and therapeutic options. *Nature medicine* 17, 1410-1422.
- Whitworth, J.A., Williamson, P.M., Mangos, G., and Kelly, J.J. (2005). Cardiovascular consequences of cortisol excess. *Vascular health and risk management* 1, 291-299.
- Wigren, M., Bjorkbacka, H., Andersson, L., Ljungcrantz, I., Fredrikson, G.N., Persson, M., Bryngelsson, C., Hedblad, B., and Nilsson, J. (2012). Low levels of circulating CD4⁺FoxP3⁺ T cells are associated with an increased risk for development of myocardial infarction but not for stroke. *Arterioscler Thromb Vasc Biol* 32, 2000-2004.

- Viiri, L.E., Full, L.E., Navin, T.J., Begum, S., Didangelos, A., Astola, N., Berge, R.K., Seppala, I., Shalhoub, J., Franklin, I.J., *et al.* (2013). Smooth muscle cells in human atherosclerosis: proteomic profiling reveals differences in expression of Annexin A1 and mitochondrial proteins in carotid disease. *Journal of molecular and cellular cardiology* 54, 65-72.
- Virmani, R., Kolodgie, F.D., Burke, A.P., Farb, A., and Schwartz, S.M. (2000). Lessons from sudden coronary death: a comprehensive morphological classification scheme for atherosclerotic lesions. *Arterioscler Thromb Vasc Biol* 20, 1262-1275.
- Witko-Sarsat, V., Rieu, P., Descamps-Latscha, B., Lesavre, P., and Halbwachs-Mecarelli, L. (2000). Neutrophils: molecules, functions and pathophysiological aspects. *Laboratory investigation; a journal of technical methods and pathology* 80, 617-653.
- Wong, K.L., Yeap, W.H., Tai, J.J., Ong, S.M., Dang, T.M., and Wong, S.C. (2012). The three human monocyte subsets: implications for health and disease. *Immunologic research* 53, 41-57.
- Wu, C.Y., Wang, K., McDyer, J.F., and Seder, R.A. (1998). Prostaglandin E2 and dexamethasone inhibit IL-12 receptor expression and IL-12 responsiveness. *Journal of immunology* 161, 2723-2730.
- Yang, Y.H., Aeberli, D., Dacumos, A., Xue, J.R., and Morand, E.F. (2009). Annexin-1 regulates macrophage IL-6 and TNF via glucocorticoid-induced leucine zipper. *Journal of immunology* 183, 1435-1445.
- Yang, Y.H., Morand, E.F., Getting, S.J., Paul-Clark, M., Liu, D.L., Yona, S., Hannon, R., Buckingham, J.C., Perretti, M., and Flower, R.J. (2004). Modulation of inflammation and response to dexamethasone by Annexin 1 in antigen-induced arthritis. *Arthritis and rheumatism* 50, 976-984.
- Yang, Y.H., Song, W., Deane, J.A., Kao, W., Ooi, J.D., Ngo, D., Kitching, A.R., Morand, E.F., and Hickey, M.J. (2013). Deficiency of annexin A1 in CD4+ T cells exacerbates T cell-dependent inflammation. *Journal of immunology* 190, 997-1007.
- Yazid, S., Norling, L.V., and Flower, R.J. (2012). Anti-inflammatory drugs, eicosanoids and the annexin A1/FPR2 anti-inflammatory system. *Prostaglandins & other lipid mediators* 98, 94-100.
- Yu, H.G., Lee, D.S., Seo, J.M., Ahn, J.K., Yu, Y.S., Lee, W.J., and Chung, H. (2004). The number of CD8+ T cells and NKT cells increases in the aqueous humor of patients with Behçet's uveitis. *Clinical & Experimental Immunology* 137, 437-443.

Yusuf, S., Hawken, S., Ounpuu, S., Dans, T., Avezum, A., Lanas, F., McQueen, M., Budaj, A., Pais, P., Varigos, J., *et al.* (2004). Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case-control study. *Lancet* 364, 937-952.

Yusuf, S., Rangarajan, S., Teo, K., Islam, S., Li, W., Liu, L., Bo, J., Lou, Q., Lu, F., Liu, T., *et al.* (2014). Cardiovascular risk and events in 17 low-, middle-, and high-income countries. *The New England journal of medicine* 371, 818-827.

Zhang, N., and Bevan, M.J. (2011). CD8(+) T cells: foot soldiers of the immune system. *Immunity* 35, 161-168.

Ziegler-Heitbrock, L., Ancuta, P., Crowe, S., Dalod, M., Grau, V., Hart, D.N., Leenen, P.J., Liu, Y.J., MacPherson, G., Randolph, G.J., *et al.* (2010). Nomenclature of monocytes and dendritic cells in blood. *Blood* 116, e74-80.

Papers

The articles associated with this thesis have been removed for copyright reasons. For more details about these see:

<http://urn.kb.se/resolve?urn=urn:nbn:se:liu:diva-114123>