T Cells and NK Cells in Coronary Artery Disease:

Longitudinal and methodological studies in humans

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.....*and nothing else matters.*

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Abstract

Coronary artery disease (CAD) is the leading cause of death worldwide and most often due to atherosclerosis. Atherosclerosis is a chronic inflammatory process that involves the arteries, including those that supply blood to the heart muscle. Although inflammation is an important contributing factor to atherosclerosis, the mechanisms are not fully understood. One mechanism contributing to atherogenesis may involve some infectious microorganisms such as cytomegalovirus (CMV). In atherosclerosis, the arterial wall becomes infiltrated with lipids followed by different types of leukocytes and inflammatory mediators (atherogenesis). Leukocytes recirculate continuously between the blood and lymphoid organs, such as lymph nodes, where the adaptive immune response is started and regulated.

The general aim of this thesis was to increase the understanding of associations between lymphocyte populations and different conditions of CAD (unstable and stable). To assess changes over time, a longitudinal follow up design was mostly used. Therefore, also perspectives of longitudinal variation were included in the thesis.

Paper I showed that flow cytometric evaluation of lymphocyte populations is a robust technique that can be used in longitudinal studies, both in clinical and research settings. It was also shown that the time of sampling over the year did not have a major impact on the findings.

In paper II, thoracic lymph nodes were investigated to assess whether CAD-associated changes were more prominent in comparison with blood. As expected, there were several major differences in lymphocyte composition between lymph nodes and blood. However, the analysis of thoracic lymph nodes did not reveal any further changes that were not detected in blood. Thus, blood is still the most reliable compartment for studies of lymphocyte populations in CAD since it is not possible to examine the local findings in the artery wall.

Natural killer (NK) cells are innate lymphocytes with both regulatory and effector functions. In paper II and III we confirmed previous findings that CAD patients have lower proportions of NK cells in blood. However, the NK subtype and cytokine profile (paper III, measured by subtype markers and intra-cellular cytokine staining) did not differ between patients and controls. During a 12-month follow-up, the proportions of NK cells increased, although not in all patients. Failure to reconstitute NK cell levels was associated with several components of the metabolic syndrome and with a persistent low-grade inflammation as measured by plasma IL-6 levels. The findings support the notion of a protective role for NK cells in inflammation.

CD4⁺ but not CD8⁺ T cells were significantly increased in patients with both unstable and stable conditions compared with healthy individuals (paper IV). Subpopulations of CD4⁺ T cells (CD4⁺CD28^{null}) have previously been associated with CAD. However, we show that CD28^{null} and CD28^{null}57⁺ cells within the CD4⁺ and CD8⁺ T cell populations were similar in CAD patients and healthy controls. Instead, CMV seropositivity was the major determinant of expanded CD28^{null} and CD57⁺ T cell fractions in both patients and healthy individuals. During the 1 year follow up the proportion of CD4⁺CD28^{null} and CD8⁺CD28^{null} cells increased in patients, which may reflect an accelerated immunological ageing occurring after the cardiac event.

Sammanfattning på svenska

Hjärtkärlsjukdom är den största bidragande orsaken till för tidig död i världen och orsakas i de flesta fall av ateroskleros. Ateroskleros är en kronisk inflammatorisk process. Artärer, bland annat de som försörjer hjärtat med syre, blir infiltrerade av blodfetter, vilket leder till en inflammatorisk reaktion med infiltration av vita blodkroppar i kärlväggen. Inflammation i sig är en starkt bidragande faktor till ateroskleros men mekanismerna är ofullständigt kända. En bidragande faktor som diskuteras är infektiösa agens, som t.ex. humant cytomegalovirus (CMV). Aterosklerotiska plack karakteriseras av infiltrat i kärlväggen bestående bl.a. av olika typer av vita blodkroppar (neutrofiler, monocyter och lymfocyter) som är en del i den inflammatoriska processen. De vita blodkropparna cirkulerar kontinuerligt mellan blodbanan (där de kan stöta på främmande agens) och olika lymfoida organ, som t.ex. lymfkörtlar, där immunsvaret både startar och regleras.

Det övergripande syftet med den här avhandlingen var att öka förståelsen för sambandet mellan olika typer av lymfocyter med olika former av hjärtsjukdom (instabil och stabil). I ett längre perspektiv skulle dessa nya kunskaper kunna användas vid diagnostisering och monitorering av hjärtsjukdom.

Eftersom vi i flera studier gjorde långtidsuppföljningar med upprepade prover var det viktigt att kartlägga hur stor den normala variationen är. Resultaten i arbete I visar att den teknik som använts ger stabila resultat över tid och att till exempel tidpunkt under året inte inverkar på resultaten. Fynden är av betydelse både i klinisk användning och i forskningsstudier.

Immunologiska reaktioner startar i lymfkörtlar och därför ville vi i arbete II undersöka om lymfocytpopulationer i lymfkörtlar kunde ge mer information än blod vid studier av hjärtsjukdom. Det fanns flera stora skillnader i fördelningen av olika lymfocytpopulationer mellan lymfkörtel och blod, men det framkom inga ytterligare skillnader mellan patienter och kontroller jämfört med i blod. Därför är undersökningar av lymfocyter i blod fortfarande den mest lämpliga metoden, eftersom det inte går att ta prov på den lokala processen i blodkärlsväggen.

I tidigare studier har vi påvisat en låg andel av "natural killer" (NK) celler i blod vid hjärtsjukdom. Detta fynd konfirmerades i arbete II och III. Vi undersökte i detalj NK cellerna avseende olika markörer och funktionella egenskaper, men kunde inte påvisa några skillnader mellan patienter och friska (arbete III). Vid uppföljningsprover på patienterna efter 12 månader hade andelen NK normaliserats hos många men inte alla patienter. De patienter vars NK celler inte återhämtat sig visade sig ha tecken på kvarstående inflammation (förhöjda nivåer av IL-6 i plasma) och även avvikelser i blodfetter och midjemått (faktorer som ingår i det metabola syndromet). Fyndet tyder på att NK celler kan ha en skyddande effekt vid hjärtsjukdom genom att motverka inflammation.

I arbete IV undersöktes T celler vid hjärtsjukdom. I likhet med tidigare studier var andelen hjälpar T celler signifikant ökad hos både instabila och stabila patienter jämfört med friska personer. Olika undergrupper av T celler har tilldragit sig stort intresse vid hjärtsjukdom, till exempel celler som saknar uttryck av CD28 (CD28 negativa). Dessa celler har tillskrivits en roll i utveckling av hjärtsjukdom, men de påverkas också av cytomegalovirus (CMV) och är markörer för immunologiskt åldrande. I detta arbete fann vi att förekomst av latent CMV infektion var den starkaste bidragande faktorn till expansion av CD28 negativa T celler. Betydelsen av detta fynd är oklar, men visar att förekomst av CMV infektion bör kartläggas vid studier av hjärtsjukdom. Under 1 års uppföljning ökade andelen CD28 negativa T celler hos patienterna. Detta kan tolkas som att CMV skyddar på åldrande av T celler hos patienter med hjärtkärlsjukdomar.

Original Publications

This thesis is based on the following articles, which are referred to in the text by Roman numbers:

- I. **Karin Backteman, Jan Ernerudh**
Biological and methodological variation of lymphocyte subsets in blood of human adults
Journal of Immunology Methods, 2007 Apr;322(1-2):20-27
- II. **Karin Backteman, Carina Andersson, Lars Göran Dahlin, Jan Ernerudh, Lena Jonasson**
Lymphocyte subpopulations in lymph nodes and peripheral blood: A comparison between patients with stable angina and acute coronary syndrome
PLoS One. 2012;7(3):e32691. doi: 10.1371/journal.pone.0032691.
- III. **Karin Backteman, Jan Ernerudh, Lena Jonasson**
NK cell deficit in coronary artery disease: No aberrations in phenotype but sustained reduction of NK cells is associated with low-grade inflammation
Clinical & Experimental Immunology. 2014 Jan;175(1):104-12. doi: 10.1111/cei.12210
- IV. **Karin Backteman, Jan Ernerudh, Lena Jonasson**
Cytomegalovirus seropositivity is a major determinant of CD28^{null} T cell expansion in patients with coronary artery disease.
Manuscript for submission to Clinical and Experimental Immunology

Abbreviations

ACS	acute coronary syndrome
APC	antigen presenting cell
APC	allophycocyanin
APC-H7	allophycocyanin cyanin 7
CAD	coronary artery disease
CCL	C-C motif ligand
CCR	C-C chemokine receptor
CD	cluster of differentiation
CMIA	chemiluminescent microparticle immunoassay
CSF	colony-stimulating factor
CTLA	cytotoxic T lymphocyte antigen
CV	coefficient of variation
CVD	cardiovascular disease
ECs	endothelial cells
ECG	electrocardiogram
EDTA	ethylenediamine tetraacetic acid
ELAM	endothelial leukocyte adhesion molecule
ELISA	enzyme-linked immunosorbent assay
FITC	fluorescein isothiocyanate
FMO	fluorescence minus one
FSC	forward-scattered light
Foxp3	forkhead box p3
GITR	glucocorticoid-induced tumor necrosis factor receptor
CMV	cytomegalovirus
HDL	high-density lipoprotein
HLA	human leukocyte antigen
IFN	interferon
ICAM	intercellular adhesion molecule
IL	interleukin
KIRs	killer immunoglobulin-like receptors
L	ligand
LDL	low-density lipoprotein
LFA	lymphocyte function-associated antigen
LN	lymph node

LPS	lipopolysaccharide
MALT	mucosa-associated lymphoid tissue
MDC	macrophage derived chemokine
MHC	major histocompatibility complex
MMP	matrix metalloproteinases
NK	natural killer (cell)
NLRs	nucleotide binding and oligomerization domain like receptor
Non-STEMI	non-ST elevation myocardial infarction
oxLDL	oxidized low-density lipoprotein
PAMPs	pathogen associated molecular antigen
PB	pacific blue
PE	phycoerythrin
PerCP	peridinin chlorophyll protein
PMA	phorbol myristate acetate
PMT	photomultiplier
PRR	pattern recognition receptor
S1PR1	sphingosine 1 phosphate receptor 1
SA	stable angina
SSC	side-scattered light
SMC	smooth muscle cells
SPSS	statistical package for the social sciences
TCR	T cell receptor
T _{FH}	Follicular helper T cells
TG	triglycerides
TGF	transforming growth factor
Th	T helper (cell)
TLRs	toll like receptor
TNF	tumor necrosis factor
Treg	regulatory T cell
VCAM	vascular cell adhesion molecule
VLA	very late activation antigen
VSMC	vascular smooth muscle cells

Introduction

This thesis discusses some immunological mechanisms and perturbations in patients with coronary artery disease (CAD), both acute coronary syndrome (ACS) and stable angina (SA), early as well as during longitudinal follow up. Some methodological aspects concerning the flowcytometry technique will also be discussed.

Atherosclerosis

Atherosclerosis is a pathological process that underlies several severe cardiovascular diseases (CVD) such as CAD, stroke and peripheral arterial disease. In 2008 the global total number of deaths was estimated to 57 millions, 17 millions were suffering from CVD and 90-95 % among them were CAD cases (Pranavchand and Reddy, 2013). In Sweden the number of deaths with acute myocardial infarction as underlying cause was 8 300 in 2012, a decrease by 40 % since 2012.

Initially atherosclerosis was considered as a cholesterol storage disease, but it is now accepted that the underlying pathological process is also a chronic inflammatory disorder affecting the arterial blood vessels. The inflammatory process involves both the innate and the adaptive immune system modulating the initiation and progression of plaque development (Hansson and Libby, 2006). The vascular changes occur mainly in the intima of middle-sized and large arteries such as aorta, carotid, femoral and coronary arteries, seldom in small arteries and never in venous vessels. Also, plaques often develop in locations with slower flow, nearby bifurcations. As the atherosclerotic plaque grows, the arterial lumen will be narrowed. This, in combination with reduced elasticity of the arterial wall, leads to a reduced blood flow and a lack of oxygen supply to the tissue. If it occurs in the coronary arteries, it can result in effort-induced chest pain named stable angina pectoris. The vascular stiffening and narrowing process will also lead to increased blood pressure (Libby, 2008).

To simplify, atherosclerotic plaques are separated into two broad categories: stable and unstable (also called vulnerable) (Neri Serneri et al., 1997). Under certain circumstances, often associated with increased inflammatory activity, the stable plaque may transform into an unstable plaque. Such a plaque may rupture and thrombogenic material such as collagen will then be exposed to the circulation and induce thrombus formation (Didangelos et al., 2009). If this occurs in the carotid artery, it can lead to stroke and similarly, if the thrombus is formed in the coronary artery, it can give rise to an acute myocardial infarction (or acute coronary syndrome, ACS) (Neri Serneri et al., 1997).

The immune system

The immune system is the defence system of the body. The main function is to distinguish between self molecules (antigens) in the own body and non-self antigens such as bacteria, viruses and fungi, as well as damaged tissue in the body. Moreover it must not react against non-harmful particles such as pollen. It is a complex system consisting of cells and soluble molecules such as complement factors and cytokines. Traditionall, the immune system is divided into innate and adaptive immunity.

Innate immunity

The innate immune system, previously known as the non-specific immune system, is the first line of defense and comprises anatomical barriers (such as skin and mucosa), cells, cytokines, complement factors and other effector proteins that can provide immediate defense against microbes. It has no memory but recognize, respond to, and kill pathogens fast. The innate cells include natural killer (NK) cells, mast cells, monocyte derived macrophages, granulocytes and dendritic cells (Abbas, 2012). These cells attack microbes that have passed through the epithelial barriers and entered into tissues or the circulation. The innate system is triggered when pattern recognition receptors (PRRs), *e.g.* toll like receptors (TLRs) and nucleotide binding and oligomerization domain like receptors (NLRs), on the innate cells recognize pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharides (LPS) on the microbes (Janeway, 2011). In response some of these cells secrete cytokines, chemokines and adhesion molecules that activate phagocytes and stimulate inflammation. The innate reactions are important in forming the adaptive immunity..

In the arterial intima, macrophages expressing PPRs mediate degradation of lipoprotein particles and apoptotic bodies. Normally, this process in itself does not lead to inflammation but in the long run, it can lead to a massive accumulation of lipid-filled macrophages, so-called foam cells, in the arterial wall.

Adaptive immunity

The adaptive immune system, previously known as the specific immune system, is composed of highly specialized cells and processes that eliminate or prevent pathogenic growth. The adaptive response is slow, initiated by the innate immune system and requires antigen presentation by specific antigen presenting cells (APC). The adaptive immune system is divided into humoral immunity mediated by B cells and antibodies with function to for example eliminate bacteria, and cell-mediated immunity mediated by T cells that for example activate infected macrophages for killing or lyse infected cells (Abbas, 2012). The characteristics of the adaptive immune response are specific recognition, clonal expansion and differentiation of lymphocytes into effector or memory cells. The memory cells allow a faster, stronger and more efficient second immune response to a previously encountered antigen. The adaptive immune system very much acts by enhancing mechanisms of the innate immune system like inflammation and phagocytosis.

B cells and T cells are derived from the same multipotent hematopoietic stem cells, and share morphology until they are activated (Janeway, 2011). B cells mature in the bone marrow and migrate via the bloodstream to the secondary lymphoid organs where they aggregate into primary follicles or germinal centers. When B cells are activated by antigen, usually with help from T cells, they proliferate and mature into memory B cells or plasma cells. The memory B cells can promptly respond to antigens resulting in a more rapid response. The function of the plasma cells is to produce large numbers of antibodies. The antibody is of the same specificity as the antigen receptor on the stimulated B cell and reacts therefore with the same antigen. The antigen and antibody form a complex that will be eliminated by phagocytes.

Immature T cells travel from bone marrow to thymus where they undergo differentiation and selection and then travel further to peripheral lymphoid tissues. There are two major functional subsets: T helper (Th) cells expressing the cell surface molecule cluster of differentiation (CD)4, and T cytotoxic cells expressing the CD8 cell surface molecule. Th cells provide help to B cells by direct surface signaling and by producing cytokines that are crucial for B cells differentiation. T cytotoxic cells mediate killing of infected cells via apoptosis by releasing granzymes and perforins in the same way as for the NK cells, which are cytotoxic cells of the immune system. In addition to cytotoxic effects NK cells may also have regulatory functions by secretion of cytokines (Yokoyama et al., 2004).

Lymph nodes

Lymphocytes continuously recirculate between the bloodstream and the secondary lymphoid organs (lymph nodes (LN), spleen and mucosa-associated lymphoid tissue (MALT)) and if they encounter an antigen they then migrate to peripheral inflammatory sites. Lymph nodes situated along lymphatic vessels are small solid structures with a size from 2 to 20 mm. In brief, a LN consists of an outer cortex and an inner medulla all surrounded by a fibrous capsule. The outer cortex contains the primary follicles, which are comprised of naïve B cells, and germinal centers, which contain stimulated B cells and memory B cells. The inner cortex contains T cells zones with T cells (mostly CD4⁺ T cells) and some dendritic cells. Also NK cells reside in the T cells area. In contrast to blood, the NK cells in LNs are CD56^{bright} (95% of all NK cells) with high cytokine production and low cytolytic activity (Fehniger et al., 2003). The lymph enters and leaves the nodes through afferent and efferent vessels, whereas naïve T and B cells enter the nodes through an artery (Figure 1) (Janeway, 2011, Abbas, 2012).

Figure 1. Schematic view of a LN. The LN consists of follicles with naïve B cells (B cell zones) and germinal centers with activated B cells and also T cell zones. From the bloodstream naïve B cells and T cells enter the LN through artery vessels whereas antigen bearing dendritic cells from lymph enter the LN through afferent lymphatic vessels. In response to antigens T cells get activated and enter the circulation via efferent lymphatic vessels whereas B cells, after being activated by T cells, migrate into germinal centers where plasma cells initiate antibody production. Plasma cells then migrate and a large portion resides in the bone marrow.

The human body has about 500 milliard (5×10^{11}) lymphocytes, in blood constituting 20-40 % of the leukocytes. Forty % of the total numbers of lymphocytes in the body exist in the LN (Blum and Pabst 2007). Approximately 1-2% of the lymphocyte pool recirculate each hour to optimize the opportunities for antigen-specific lymphocytes to find their specific antigen within the secondary lymphoid tissues (Blum and Pabst, 2007).

The initiation of an immune response starts in LN when antigen-loaded dendritic cells enter the LN through lymphatic vessels. When naïve lymphocytes in the LN encounter antigens the differentiation phase into effector cells starts (Figure 2) (Deenick et al., 2011). The lymphocytes upregulate integrins and ligands, such as E- and P-selectin, as well as lymphocyte function-associated antigen (LFA)-1 or very late activation antigen (VLA)-4. Meanwhile adhesion molecules needed for passage from blood to LNs are down-regulated; CD62L (the L-selectin specific for ligands on endothelial cells in LNs) and CC-chemokine receptor (CCR)7. Taken together these changes make it possible for effector lymphocytes to change their route and migrate to sites of inflammation (Janeway, 2011, Abbas, 2012).

Figure 2. Schematic view of T cell and B cell activation in LN. CD8⁺ and CD4⁺ T cells become activated when they recognize peptides bound to MHC class I or class II molecules respectively, on dendritic cells. Follicular helper T cells (T_{FH}) migrate further to the B cell zone and interact with activated B cells which in turn differentiate into plasmablasts or enter the germinal zone where B cells differentiate into plasma cells or memory B cells.

Lymphocyte subsets

Blood is composed of plasma (about 55 %), erythrocytes (about 45 %), platelets and leukocytes and constitutes approximately 8 % of the total body weight. Five major types of leukocytes are classified depending on granules (neutrophils, eosinophils and basophils) or not (monocytes and lymphocytes) in the cytoplasm. Further, lymphocytes are divided into Th cells, T cytotoxic cells, B cells and NK cells. Only 2 % of the total lymphocytes exist in the bloodstream, of which 60 % recirculate continuously between the bloodstream, primary lymphoid organs and secondary lymphoid organs such as spleen, Peyer's patches and LN (Blum and Pabst, 2007).

T helper cells (CD4⁺) expressing transmembrane glycoproteins CD3 and CD4 play a central role in immune protection through their capacity to help B cells make antibodies, inducing macrophages to develop enhanced microbicidal activity, recruiting several cell types including granulocytes to sites of infection and inflammation, and producing cytokines and chemokines for different kinds of immune responses. CD4⁺ Th cells are further subdivided depending on their cytokine profile and function, the established subpopulations being Th1, Th2, Th17 and regulatory T cells (Treg) (Figure 3).

Figure 3. A simplified view of cytokines needed for naïve T cell differentiation into different Th subsets, their cytokine secretion, main functions and presumed role in atherosclerosis. In addition natural Tregs arise in the thymus.

The majority of lymphocytes in human atherosclerotic plaques are CD4⁺ cells producing different cytokines with pro- or anti- inflammatory effects. Most of the CD4⁺ cells are believed to represent an interleukin (IL)-12 induced Th1-type mainly producing interferon (IFN)- γ , a phenotype that is associated with atherosclerosis progression and plaque destabilization. Only few cells are Th2 type producing IL-4 that theoretically may decrease the plaque burden by preventing the production of IFN- γ and thereby, the Th1 cell differentiation (Packard et al., 2009, Wigren et al., 2012b). Other Th2 cytokines, for example IL-5, have in experimental studies shown to stimulate B cells to produce antibodies with protective effects in atherosclerosis (Binder et al., 2004).

CD4⁺ regulatory T cells (Tregs) constitute a subpopulation of CD4⁺ Th cells that suppress the activation of effector T cells through several mechanisms including secretion of IL-10 and transforming growth factor (TGF)- β and through inhibition of APCs functions (Ait-Oufella et al., 2009). Natural Tregs develop in thymus and recognize self-antigens or foreign antigens. Induced Tregs are induced in the periphery from naïve CD4⁺ cells (Figure 2). There is no reliable marker to distinguish natural (thymic) from induced Tregs. Human Tregs are classically characterized by the expression of the transcription factor Forkhead box p3 (Foxp3) or high surface expression of the IL-2 receptor α -chain (CD25). However, none of these markers by itself discriminate suppressive Tregs from activated cells since both markers are expressed in activated cells. It was shown that CD25^{bright} cells with a slightly lower CD4 expression (CD4^{dim}) represented true Tregs as shown by additional marker expression and functional suppression studies (Mjosberg et al., 2009). Other suggested markers for Tregs include CD127^{low/negative}, cytotoxic T lymphocyte antigen (CTLA)-4, glucocorticoid-induced tumor necrosis factor receptor (GITR), and CD45RA, in different combinations with or without CD25 and Foxp3 (Liu et al., 2006, Brusko et al., 2008, Miyara et al., 2009). In experimental studies it has been shown that Tregs have a protective role in murine atherosclerosis (Ait-Oufella et al., 2006, Mor et al., 2007). In human atherosclerotic plaques the numbers of Tregs, defined as Foxp3 positive T cells, are low (1-5 % of T cells) compared to other chronically inflamed tissue (up to 25 % of T cells) suggesting an increased arterial inflammation due to impaired local tolerance (de Boer et al., 2007). Also in blood decreased levels of Tregs have been reported in patients with ACS (Mor et al., 2006, Mor et al., 2007). In addition a protective role of Tregs in development of atherosclerosis has been suggested (Wigren et al., 2012a). On the other hand, both Ammirati et al and Wigren et al showed that there were no correlations between number of Tregs and carotid intima media thickness in population-based cohorts (Ammirati et al., 2010). These contradictory results may depend in part on the gating strategies for defining Tregs but also illustrate that Tregs are involved in plaque destabilization rather than in atherosclerosis development.

T cytotoxic cells (CD8⁺) expressing transmembrane glycoproteins CD3 and CD8 recognize and respond to foreign peptides and kill both infected and tumor cells. CD8 cells are major effector cells of the adaptive immune system recognizing target cells by binding to antigens bound to MHC I, which is present on the surface of all nucleated cells. When differentiated effector CD8⁺ T cells encounter foreign antigens, the infected cells get killed by cytolytic mechanisms. Apoptosis is induced either by cytotoxic effects from perforin and granzymes or by binding of Fas ligand (L) to its receptor on the infected cell (Abbas, 2012).

In atherosclerosis, CD8⁺ T cells are present only at low numbers in early lesions but have been described to be the dominating T cell type in advanced human lesions (Gewaltig et al., 2008).

The role of CD8⁺ cells in atherosclerotic plaque is yet unclear although in experimental animal models increased numbers and functions promote inflammation, which could be explained by their cytotoxic killing function of vascular cells (Ludewig et al., 2000, Gewaltig et al., 2008). It has also been reported that increased levels of CD8⁺ T cells in patients with CAD are associated with human cytomegalovirus (CMV) infection (Jonasson et al., 2003). Moreover, in a recent population-based prospective study, the frequencies of CD8⁺ T cells were found to be associated with increased risk of coronary events (Kolbus et al., 2013).

$\gamma\delta$ T cells constitute a small subpopulation of T cells (about 1-5 % in blood) expressing the transmembrane glycoproteins γ and δ in contrast to most of the T cells that express $\alpha\beta$ receptors. Most of the $\gamma\delta$ T cells do not express CD4 or CD8. $\gamma\delta$ T cells are more common at sites of chronic inflammation and in mucosal tissues such as gastrointestinal mucosa and skin but can vary widely between different kind of tissues (Vanderlaan and Reardon, 2005). Some $\gamma\delta$ T cells have been detected in the intima of human atherosclerotic lesions, especially in the early stages of lesions formations (Millonig et al., 2002). However, their role in atherosclerosis progression is still unclear; even antiatherogenic properties have been suggested (Galkina and Ley, 2009, Packard et al., 2009, Profumo et al., 2012).

B cells (CD19⁺) expressing CD19 but lacking CD3 are the only lymphocytes capable of producing antibodies. B cells express membrane-bound immunoglobulin receptors that recognize foreign antigens, after which the activation process can start. Stimulated and differentiated B cells produce antibodies that in their secreted form bind to antigens. In atherosclerosis, B cells are considered to mediate protective immunity by eliminating atherosclerosis-related antigens, such as oxidized lipoprotein particles (Packard et al., 2009).

In the atherosclerotic intima, B cells are only occasionally detected (Galkina and Ley, 2009) but early plaques contain large amounts of IgM and IgG (Yla-Herttuala et al., 1994).

NK cells (CD56⁺) expressing CD56 but lacking CD3 are lymphocytes that belong to innate immunity. NK cells, also termed “large granular lymphocytes”, are produced in the bone marrow. They can be found in tissues but are mainly present in the circulation where they comprise about 5-15% of the total lymphocyte fraction. The main function is to recognize and kill virus-infected self cells and some tumor cells without previous sensitization. If NK cells bind to uninfected self cells, the killer immunoglobulin-like receptors (KIRs) on the NK cells recognize MHC class I and send inhibitory signals to the NK cells that prevent activation and killing the self cells. If the NK cells bind to virus-infected or tumor cells, the MHC expression will be reduced and the inhibitory signals from the KIR's will be reduced leading to activation and cytotoxic actions of the NK cells. NK cells perform killing via the same effector molecules as CD8⁺ T cells, such as granzymes/perforin and FasL. NK cells also secrete cytokines, predominately IFN- γ (Yokoyama et al., 2004, Backteman et al., 2014), which enhances the killing function.

Further, NK cells can be divided into two main functional subsets depending on the density of CD56. NK cells with high CD56 expression (CD56^{bright}) are immunoregulatory and more cytokine producing than NK cells with low CD56 expression (CD56^{dim}). CD56^{dim} cells on the other hand are more potent mediators of cytotoxicity than CD56^{bright} (Cooper et al., 2001, Pranavchand and Reddy, 2013). In blood CD56^{bright} cells make up of less than 10% of NK cells, whereas in LN the fraction is about 30 % of NK cells (Cooper et al., 2001, Backteman et al., 2012). NK cells can also be subdivided based on the expression of other surface receptors. CD57 on NK cells acts as a differentiation marker where CD56^{bright} cells that do not

express CD57 differentiate via $CD56^{dim}CD57^-$ to $CD56^{dim}CD57^+$. These highly differentiated $CD56^{dim}CD57^+$ cells are associated with normal ageing but also with increased responsiveness to target cells and decreased response to cytokines (Chidrawar et al., 2006, Gayoso et al., 2011). In human atherosclerotic plaques, NK cells can be detected particularly in the shoulder regions (see below). Experimental animal studies have shown that NK cells activated by cytokines such as $IFN-\alpha/\beta$, IL-12, IL-15 and IL-18 may contribute to lesion progression (Packard et al., 2009). In patients with CAD, the proportions of NK cells in blood have been shown to be significantly lower than in healthy controls (Jonasson et al., 2005).

Identification of lymphocyte subpopulations

A large number of cell surface markers are used to classify subpopulations of lymphocytes. Below a number of markers used in this thesis are presented.

CD69, a membrane bound receptor, is an activation marker expressed on very early activated T, B and NK cells and platelets. It is expressed already 30-60 min after T cell receptor (TCR) stimulation and with a decline already after 4-6 hours. Twenty-four hours after stimulation, CD69 is barely detectable (Hosono et al., 2003). In blood the numbers of $CD69^+$ cells are few but may be increased in acute inflammatory diseases (Evans et al., 2011, Jost et al., 2011). On the other hand CD69 is frequently more expressed on T cells in LN, suggested to promote lymphocyte retention (Shiow et al., 2006).

HLA DR is an activation marker expressed on late activated T and NK cells. It is constitutively expressed on APCs including dendritic cells, monocytes/macrophages and B cells. In blood the numbers of $HLA\ DR^+$ T cells are few but they may be increased in acute and chronic inflammatory diseases (Evans et al., 2011, Jost et al., 2011).

The chemokine receptor 4 (CCR) 4 is a receptor for CCL17/TARC (C-C motif ligand) 17 / (thymus and activation-regulated chemokine) and CCL22 (macrophage-derived chemokine/MDC) among T cells expressed on polarized Th2 cells (Nagira et al., 1998) and on a subset of Tregs (Ishida and Ueda, 2006). CCR4 expression is modulated by cytokines, for instance $TGF-\beta$ enhances CCR4, whereas $IFN-\alpha$ inhibits CCR4 expression (Sallusto et al., 1998, Sozzani et al., 1999, Syrbe et al., 1999). CCR4 plays an important role in recruitment of T cells to inflamed tissue. Since it has been suggested that T cells expressing CCR4 secrete IL-4 (Rivino et al., 2004), CCR4 could be used as a surrogate marker for Th2.

IL-18R, a member of the TLR superfamily, is selectively expressed on the type 1 lymphocyte and NK lineages (Chan et al., 2001, Borzychowski et al., 2005, Kang et al., 2007). Inflammatory cytokines (IL-1 β , tumor necrosis factor (TNF) and IL-12) enhance the expression of IL-18R on NK cells and T cells and thereby increase the responsiveness to IL-18. IL-18 mediated process may contribute to chronic inflammatory disorders such as rheumatoid arthritis (Gerdes et al., 2002). Whether this process also applies to human atherosclerosis is not yet fully clarified.

CD57, a human natural killer-1 glycoprotein, was at the beginning identified as a marker for NK cells (Abo and Balch, 1981, Abo et al., 1985). Subsequently it was also found to be expressed on about 15 % of $CD8^+$ T cells while it is also shown on $CD4^+$ cells but at much lower rates than for $CD8^+$ cells (Manara et al., 1988, Hebib et al., 1998, Strioga et al., 2011). NK cells expressing CD57 are suggested to be highly differentiated and cytotoxic (Chidrawar

et al., 2006). CD8⁺CD57⁺ cells with or without loss of CD28 are associated with senescence and late differentiation.

CD94/NKG2a complex, NKB1 and NKAT2. NK cells express an own repertoire of activating and inhibitory receptors including KIR that binds to MHC class I on target cells. The combination of CD94 and NKG2a constitute a complex with inhibiting functions on NK cells when recognizing HLA-E on target cells. NKB1 and NKAT2 are KIRs with inhibitory functions, recognizing HLA-B and HLA-C subtypes, respectively, on target cells.

The balance between inhibitory and activation signals determine the signal for inhibition or activation and lysing of the target cell. If inhibitory receptors recognize HLA without any activation receptor-ligand interactions, the target cell will not be lysed. On the other hand if an activation receptor binds to their ligand on the target cell, without any inhibitory signal from HLA, the target cell will be lysed. The first is an example concerning normal self cells and the later example concerns virus-infected or tumor cells with down-regulated HLA class I expression. If both inhibitory receptors binds to HLA and activation receptors bind to their ligands, the target cell will not be lysed if the inhibitory signal from HLA I is stronger than the activation signal, while it will be lysed if the activation signal is stronger (Shereck et al., 2007, Farag et al., 2002).

CD28 is a co-stimulatory receptor that interacts with B7 (CD80 and CD86) on APC. CD28 is required together with the TCR complex to obtain signals for full T cell activation and clonal expansion (Abbas, 2012). In the circulation, normally >90 % of CD4⁺ T cells and about 50 % of CD8⁺ T cells express CD28 whereas <10 % of all T cells in human plaques express CD28 (de Boer et al., 1997). 28^{null} T cells show pro- inflammatory properties secreting IFN- γ and TNF, and they are increased with age and under inflammatory conditions (Weyand et al., 1998, Liuzzo et al., 2007). This population may be cytotoxic against endothelial and smooth muscle cells (SMC) and thereby promote plaque instability (Pryshchep et al., 2006).

Cytokines

Cytokines are small proteins (15-20 kDa) produced by the immune system in response to microbes and other antigens and are involved in both the innate and the adaptive immunity. Cytokine are also produced and have actions outside the immune system. Roughly, cytokines can be divided into interleukins, (growth factors for lymphocytes and important for the immune response), chemokines (regulating leukocyte migration), IFNs (with ability to interfere with viral replication in host cells), colony-stimulating factors (CSF) (that support differentiation of hematopoietic cells) and general factors such as TGF (with ability to regulate immune responses) (Ait-Oufella et al., 2011). Cytokines can induce a variety of effects like cell growth, differentiation, chemotaxis, activation cytotoxicity and regulation of immunity. Depending on how cytokines act they can be; autocrine when they act on the same cell that secreted the cytokine; paracrine when they act on nearby cells and; endocrine when they act on cells at a different site as they are produced. Further, cytokines are often pleiotropic, meaning that one cytokine can have many different effects on different cell types, and they are often redundant meaning that many different cytokines have similar or identical functional effects.

In atherosclerosis, cytokines are thought to have important roles in the development and regulation of the process. Examples of proinflammatory and anti-inflammatory cytokines are

given below. Th1 cytokines are thought to promote the development and progression of the disease whereas some Th2 cytokines may have anti-atherogenic effects. The role of IL-17 and other Th17 cytokines is not settled, although a pro-atherogenic role has been suggested (Olson et al., 2013). Different cytokines are detected in atherosclerosis-prone vessels; some of them atheroprotective and some proatherogenic. The proatherogenic effects involve increased endothelial permeability, upregulation of adhesion molecules, downregulation of thrombomodulin and decreased production of collagen (Hansson and Libby, 2006). The anti-atherogenic effects involve fibrous building via collagen synthesis and plaque stability via dampened protease activity (Ait-Oufella et al., 2011).

Cytokines with proatherogenic effects

IL-6 produced by several cells like macrophages, SMC and endothelial cells (ECs) is normally defined as a pro-inflammatory cytokine, but in atherosclerosis, studies suggest that IL-6 also can act anti-inflammatory since IL-6 can induce the synthesis of IL-1ra and release of soluble TNF receptors, which reduce the activity of inflammatory cytokines (Galkina and Ley, 2009, Ait-Oufella et al., 2011).

IL-15 is a proinflammatory cytokine produced by neutrophils, monocytes and macrophages and it is expressed by inflammatory cells in atherosclerotic plaques (Gokkusu et al., 2010). The effector functions of IL-15 are inducing proliferation of mature T cells, generation of cytotoxic T cells and stimulation of cytokine production (Fehniger and Caligiuri, 2001, Houtkamp et al., 2001). IL-15 also has a distinct role in NK cell activation as an NK cell survival factor. In experimental animal models, an upregulation of IL-15 expression has been shown to increase formation of atherosclerotic lesions (Boyiadzis et al., 2008, Gokkusu et al., 2010).

Tumor necrosis factor (TNF) is a cytokine produced by several cell types such as monocytes, macrophages, T cells, B cells and NK cells. It is pleiotropic and has pro-inflammatory effects in atherosclerosis and other metabolic and inflammatory disorders such as obesity and insulin resistance. For instance, in atherosclerosis, TNF promotes adhesion molecule expression and increases collagen breakdown in fibrous caps (Dixon and Symmons, 2007). On the other hand, in experimental studies on mice, the results are contradictory probably depending on the underlying mechanisms of atherogenesis. (Tedgui and Mallat, 2006).

Interferon- γ (IFN) is produced by Th1 cells, but also by cytotoxic T cells and NK cells. It is pleiotropic and has pro-inflammatory effects in atherosclerosis development. IFN- γ producing cells play an important role in autoimmunity, transplant rejections and in the defence against viruses and intracellular bacteria. IFN- γ is involved both in early and late stages of atherosclerosis by promoting the recruitment and activation of T cells and macrophages, leading to increased levels of pro-inflammatory cytokines and pro-thrombotic mediators. In experimental models, IFN- γ inhibits endothelial cell proliferation, inhibits vascular smooth muscle cell (VSMC) proliferation and differentiation, and decreases collagen production by SMC, leading to reduced plaque collagen and enhanced lesion development (Hansson and Libby, 2006, Tedgui and Mallat, 2006, Ait-Oufella et al., 2011).

IL-17A belongs to the family of pro-inflammatory cytokines, has pleiotropic functions and is produced by several cell types including Th17 cells. Th17 cells are important in the defence

against extracellular bacteria and fungi and in conjunction with autoimmunity (von der Thusen et al., 2003, Ait-Oufella et al., 2011). The role of IL-17 in atherogenesis is still unclear although Eid et al demonstrated that CD4⁺ cells isolated from human atherosclerotic coronary vessels expressed both IL-17 and IFN- γ . These cytokines acted synergistically to induce proinflammatory response in VSMC (Eid et al., 2009).

Cytokines with anti-atherogenic effects

IL-10 is a pleiotropic cytokine with anti-inflammatory effects, for example inhibiting Th1, Th2 and Th17 responses. It is produced by endothelial cells, SMC, macrophages, monocytes, B cells and T cells, in particular Tregs. IL-10 is mostly associated with atheroprotective properties. For example, experimental studies show that low levels of IL-10 are associated with increased infiltration of inflammatory cells and plaque development (Tedgui and Mallat, 2006, Ait-Oufella et al., 2011). It has also been suggested that IL-10 inhibits apoptosis, thereby leading to plaque stability (Andersson et al., 2010).

The interpretation of IL-10 levels in the circulation may, on the other hand, be difficult. It has been suggested that high IL-10 levels in the circulation are associated with atherosclerotic plaque progression, plaque instability and poor prognosis (Malarstig et al., 2008, Patel et al., 2009). High IL-10 levels in plasma may be merely a marker of an on-going inflammatory process

IL-13 is produced mainly by Th2 cells, but also by B cells, macrophages and NK cells, and has anti-inflammatory effects in the context of atherosclerosis. Th2 cells producing IL-13 are important in the defence against extra-cellular parasites and are involved in allergic disease. In atherosclerosis it has been suggested that IL-13 plays a role to dampen the progression of atherogenesis and promote plaque stabilization (Tedgui and Mallat, 2006, Cardilo-Reis et al., 2012).

Development of atherosclerosis

The arteries and veins consist of 3 main layers; adventitia, media and intima with a surrounding layer of muscles, which help contract and expand the vessels. The adventia is made of collagen tissue, nerves and small blood vessels and it also contains fibroblasts and mast cells. The adventitia is the thickest layer in veins while the media is the thickest layer in arteries. The media layer consists of multiple layers of SMC surrounded by elastic tissue. The intima is the thinnest layer consisting of a single layer of endothelial cells and in arteries, a thin layer of connective tissue (Figure 4).

Figure 4. The arterial wall consists of 3 main layers; intima, media and adventitia.

The atherosclerotic plaque building process may start already in childhood and progresses during the entire life. The early lesions are called fatty streaks and are located in the arterial wall just beneath the endothelium. The fatty streaks form a pure inflammatory lesion consisting of lipid-filled macrophages, so-called foam cells (Figure 5A). These lesions do not cause symptoms but they can progress into mature atherosclerotic plaques or they can disappear with time (Ross, 1999, Hansson and Libby, 2006). The process is believed to be the same regardless of race, sex or geographic location. However, the development of plaque building is faster in patients with risk factors such as diabetes mellitus, smoking, obesity, hypertension and genetic predispositions (Insull, 2009), see also “Risk factors in atherosclerosis” below.

Atherosclerotic plaques are often separated into two broad categories: stable and unstable (also called vulnerable). The histopathology of these two lesions is briefly described below.

Stable plaques (Figure 5 B), consist of a relatively large fibrous cap containing multiple layers of SMC, fibroblasts, collagen and elastin. Lymphocytes, macrophages and dendritic cells may also be found in the fibrous cap in variable numbers. Beneath the fibrous cap, there is a relatively small necrotic core consisting of lipids, foam cells and debris. Calcifications may also be detected in the plaque (Aukrust et al., 2007, Galkina and Ley, 2009). A stable plaque can transform into an unstable plaque if the inflammatory process accelerates, however the mechanisms behind this transformation are not fully clarified. One mechanism that has been discussed throughout the years is the activation of proteolytic enzymes such as matrix metalloproteinases (MMP), in particular MMP-9 that may also promote loss of endothelial cells (Galis et al., 1994, Loftus et al., 2000). Stable plaque may be asymptomatic or cause effort-induced angina pectoris if they narrow the lumen of the coronary artery more than 70%.

Unstable plaques (Figure 5 C) consist of a relatively larger necrotic core, often comprising > 40 % of the plaque volume, and a thinner fibrous cap, compared with stable lesions. It is generally considered that the thinner the fibrous cap is, the greater is the risk of rupture. The fibrous cap contains fewer cells and less collagen and elastin and more inflammatory cells such as macrophages, lymphocytes, dendritic cells and mast cells. The inflammatory cells are primarily localized in the shoulder regions just below the rupture-prone zone. The predominant lymphocyte population in atherosclerotic infiltrates is T cells of which most are Th1, while B cells are infrequently detected (Weyand et al., 2008).

The definition of a plaque rupture is an area of fibrous cap disruption whereby the overlying thrombus is in continuity with the underlying necrotic core (Virmani et al., 2000).

Three different types of disruption in coronary lesions are considered to give rise to acute coronary syndromes. First, microscopic areas of endothelial erosions may uncover the subendothelial collagen leading to platelet adhesion and aggregation. Second, new microvessels can disrupt within the plaque. The third and the most common type is a rupture of the fibrous cap. It occurs primarily at sites characterized by a thin fibrous cap and large amounts of macrophages and T cells (van der Wal et al., 1994, Hansson, 2005). All types of plaque disruptions result in thrombus formations that more or less will block the blood flow and thereby cause ischemia and eventually an acute coronary syndrome (Hansson and Libby, 2006) (Figure 5 D).

Figure 5. Schematic view of different steps in plaque development. **A** Initially LDL infiltrates the artery and is retained in the intima where they may be modified by oxidation and enzymes forming oxLDL. These lipids activate endothelial cells to produce adhesion molecules. Circulating leukocytes bind to adhesion molecules on the activated endothelial cells and migrate into the vessel wall. Monocytes differentiate into macrophages that take up oxLDL and accumulate into foam cells. T cells get activated when interacting with macrophages or dendritic cells and in response to cytokines. The plaque building develops either into **B** a stable plaque comprised of a thicker fibrous cap or **C** an unstable plaque with a thinner fibrous cap and a relatively larger necrotic lipid core. Stable plaque may remain stable or develop into unstable if proteinases degrade the fibrous cap. **D** Erosion or disruption of the fibrous cap in unstable plaque results in thrombus formations leading to arterial occlusion.

Inflammation in atherosclerosis

Inflammation is an important contributing factor for plaque development and today atherosclerosis is regarded as a chronic inflammatory disease where both innate and adaptive immune responses play important roles (Packard et al., 2009).

Exactly what starts the progression of plaque development is not yet clarified but studies of atherosclerotic plaque building in experimental mice models have elucidated the steps in the process. In brief, low density lipoprotein (LDL) in the blood, enter the artery wall and adhere to matrix proteins where they may be modified by oxidation and aggregation (Tabas et al., 2007). These modified lipids activate the endothelial cells to express proinflammatory molecules including adhesion molecules like P-selectin, E-selectin, intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1. Thereby, immune cells like monocytes, T cells, B cells, NK cells and dendritic cells attach and migrate into the vessel wall (Figure 5 A) (Libby and Theroux, 2005). Oxidized low-density lipoprotein (oxLDL) is engulfed by the macrophages, which are then transformed into foam cells. This foam cell accumulation is named the “fatty streak”. Macrophages and T cells are localized particularly in the shoulder region, where the plaque grows. After entering the arterial wall also T cells will become activated by interacting with APC, in particular macrophages and dendritic cells. The majority of the T cells are CD4⁺ (Jonasson et al., 1986, Stemme et al., 1992) and TCRαβ⁺ which differentiate in to Th1 (Stemme et al., 1992, Methe et al., 2005, Bui et al., 2009) and together with macrophages and mast cells they contribute to the inflammatory response and plaque progression in the arterial wall by secreting cytokines such as IFN-γ, IL-1, IL-2, IL-6, TNF and growth factors that promote the migration and proliferation of SMC (Frostedgard et al., 1999, Methe et al., 2005, Bui et al., 2009). Also a substantial number of CD8⁺ cytotoxic T cells are located in the atherosclerotic plaques, (Jonasson et al., 1986, Zhou and Hansson, 1999) whereas NK cells are detected to a minor extent. B cells and plasma cells are located deeper in the layer of the plaque and in humans only in advanced plaques (Aubry et al., 2004, Robertson and Hansson, 2006). B cells divided into B1 and B2 seem to have different functions in atherosclerotic plaques. B1 cells seem to act atheroprotectively via release of antibodies against oxLDL whereas a recent experimental report suggests that B2 cells act in a proatherogenic manner stimulating T cell activation and Th1 polarization (Hilgendorf et al., 2014).

Risk factors in atherosclerosis

The development of atherosclerotic plaque has been strongly related to risk factors such as hypercholesterolemia, diabetes, hypertension, smoking, obesity, physical inactivity, psychosocial stress, age, male gender, low socio-economic status and some infections. Some risk factors can be modified but others such as age and male gender cannot be modified. The main risk factors are age, male gender, smoking, hypertension and hyperlipidemia of which hypertension and hyperlipidemia also increase by age. However, there are large individual differences. For instance, it is possible to get an infarction without any of the classical risk factors and in the same way it is possible to have many risk factors without getting a myocardial infarction (Libby, 2008).

Some of the classical risk factors will also affect the inflammatory process. For instance, obesity *per se* is associated with increased levels of proatherogenic cytokines TNF, IL-6, IL-1 and CCL2 and decreased levels of antiatherogenic adiponectin (mediator from fat cells)

leading to a chronic low-grade activation of the immune system (Arita et al., 1999, Tilg and Moschen, 2006).

The metabolic syndrome is a term that involves risk factors like decreased levels of high density lipoprotein (HDL), increased levels of triglycerides (TG), hypertension, increased waist circumference and glucose intolerance. These risk factors tend to occur together and at least 3 risk factors are needed for classification of the metabolic syndrome. The metabolic syndrome is associated with increased risk of both CVD and type 2 diabetes (Grundy, 2004).

Smoking is a risk factor for atherosclerotic vascular disease that has been known for long. Nicotine and reactive aldehydes in cigarettes promote endothelial dysfunction and vascular smooth muscle cell proliferation. Regardless of acute, chronic or passive smoking, the endothelial cells will be more or less affected (Mercado and Jaimes, 2007). Other negative effects include enhanced oxidative modification of lipoproteins and increased platelet adhesiveness.

Different kinds of psychological stress are also associated with an increased risk of CVD and atherosclerosis (Lu et al., 2013). It has been proposed that the association between stress and atherosclerosis is mediated via inflammation. Several studies have reported a correlation between plasma levels of proinflammatory cytokines and psychological factors, in particular depression (Halaris, 2013).

Cytomegalovirus (CMV)

Both bacteria and virus have been suggested as infectious organisms that can contribute to atherogenesis by infecting vascular cells and in that way activate an immune response. Infectious agents that have been associated with CVD in epidemiological studies and also have been detected in plaque tissue include porphyromonas gingivalis, chlamydia pneumonia, herpes simplex virus and CMV (Epstein et al., 2000, Xenaki et al., 2009, Borgeson et al., 2011).

CMV is an intracellularly persistent β -herpesvirus that is present in approximately 50% of the adult people and 90% of the elderly over 85 year of age (Wikby et al., 2002). Transmission of CMV can be through placental transfer, breast feeding, blood transfusion, sexual contact, saliva or transplantation of organs or stem cells (Sia and Patel, 2000). For healthy individuals, the primary infectious symptoms are modest while in immunosuppressed patients CMV may pose a particular threat. Once infected, the virus resides in cells of the myeloid lineage and the infection is lifelong. When the immune system is activated and myeloid cells differentiate into macrophages in response to inflammation, infection or stress, CMV can reactivate and start replicating (Mutimer et al., 1997, Kutza et al., 1998, Prosch et al., 2000). CMV can also reside in endothelial cells and when activated give rise to elevated levels of ICAM-1, VCAM-1 and endothelial leukocyte adhesion molecule (ELAM)-1, which enhance the migration across the endothelium of circulating leukocytes, for instance CMV infected monocytes (Sedmak et al., 1994, Burns et al., 1999). CMV has also been proven to cause increased levels of acute phase proteins and cytokine production (Epstein et al., 2000, Borgeson et al., 2011). CMV infection of endothelial cells also causes expression of von Willebrand factor resulting in platelet activation and aggregation, which may lead to thrombosis formation (Soderberg-Naucler, 2006).

It has been reported that CMV seropositive individuals show an altered composition of different T cell subsets, with an increased number of CD4⁺ and CD8⁺ cells lacking CD28 but expressing CD57. Primary CMV infection and ageing, but also chronic inflammatory diseases such as rheumatoid arthritis and vasculitis, may contribute to this expansion of T-cell subsets (Wikby et al., 2002, Fasth et al., 2004, Litjens et al., 2011, Eriksson et al., 2012).

Cellular senescence

Cellular senescence is an irreversible loss of the ability of cells to divide. In general there are two types of cell senescence, replicative senescence and stress-induced premature senescence. Replicative senescence is a form of ageing that occurs with exhaustion of proliferative lifespan over time. Stress-induced premature senescence occurs after external stimuli, *e.g.* oxidizing agents and radiation (Wang and Bennett, 2012).

Lymphocyte ageing and differentiation

Immune senescence is a kind of age-associated change in the immune system including a substantial decrease in certain lymphocyte subsets and functions. This process starts already after the puberty but accelerates after around 60 years of age (Larbi et al., 2008, Wikby et al., 2008).

Naïve CD4⁺ cells shows both a decreased responsiveness to TCR stimulation and a decreased helper function for antibody production by B cells with age (Weng, 2006). On the other hand memory CD4⁺ T cells are long-lived and remain competent throughout life (Swain et al., 2005). Among CD8⁺ T cells, there is a shift of certain subsets with increasing age. The numbers of naïve CD8⁺ CD28⁺ cells decrease whereas numbers of differentiated CD8⁺ CD28⁻ cells increase (Wikby et al., 2008). In parallel to T cells, naïve B cells are also decreased with age, which leads to an increased proportion of differentiated antigen-experienced B cells (Larbi et al., 2008). NK cells in total do not change with increasing age. However, CD56^{bright} NK cell levels are decreased while CD56^{dim} NK cell levels are preserved or increased and also, the final differentiation stage of CD56^{dim}57⁺ NK cells is increased. Decreased numbers of NK cells in elderly people are associated with decreased NK cell function and further associated with increased incidence of infectious diseases (Chidrawar et al., 2006, Gayoso et al., 2011). Furthermore it has been shown that elderly people with atherosclerosis have low cytotoxicity per NK cell without increased number of NK cells (Bruunsgaard et al., 2001).

Ageing of vessels

Vascular ageing involves intimal and medial thickening as well as gradual loss of arterial elasticity, resulting in vascular stiffness (Jani and Rajkumar, 2006). Aged vessels show reduced numbers of medial VSMC, increased collagen deposition, and fracture of the elastin lamellae, which may lead to vessel dilation and increased lumen size (Zieman and Kass, 2004). Except from ageing, hypertension *per se* can stimulate the collagen production leading to increased vessel stiffness, endothelial dysfunction, elevated expression of proinflammatory molecules and increased uptake of plasma lipoproteins (Hashimoto et al., 1991). All this together may contribute to increased expression of leukocyte adhesion molecules on the endothelial cells. Furthermore, aged vessels secrete increased amounts of cytokines with proinflammatory effects. This may predict enhanced migration of monocytes and lymphocytes into the vessel wall. Taken together, these changes are thought to be one contributing factor for development of CVD amongst ageing individuals (Wang and Bennett, 2012).

Aims and Hypothesis

General aims

The general aim was to increase the understanding of lymphocyte subpopulations in CAD and their possible role in disease development. This knowledge might in a longer perspective lead to novel diagnostic and monitoring tools as well as to identify treatment targets.

In CAD, previous studies on circulating lymphocyte populations have often been cross-sectional, while longitudinal studies are scarce. In order to perform longitudinal studies, normal biological and methodological variation in lymphocyte populations should be known. Moreover, lymphocytes in blood constitute only a minor portion of all lymphocytes. Therefore, it can be of value to investigate if aberrations in lymphocyte populations in CAD are more pronounced in lymph nodes than in blood. Finally, the presence of latent CMV infection is known to induce persistent alterations in lymphocyte populations, in particular CD28^{null} T cell populations. The impact of CMV infection on lymphocyte aberrations in CAD is therefore important to evaluate.

Specific aims

The specific aim of paper I was to determine the total variation of major lymphocyte populations over time in healthy individuals and to estimate the effects of methodological as well as biological variation.

The specific aim of paper II was to clarify differences and similarities of different lymphocyte subpopulations in blood compared to LN and to evaluate if patients with CAD show lymphocyte aberrations in LN that are not detected in blood.

The specific aim of paper III was to assess whether low NK cell levels in CAD patients represented a permanent phenomenon or fluctuated over time depending on clinical disease activity or other factors. An additional aim was to find out if the NK cell status, based on NK cell phenotyping and cytokine profile, differed between patients with non-ST elevation myocardial infarction (non-STEMI), SA and controls.

The specific aim of paper IV was to longitudinally assess the proportions of CD28^{null} and 28^{null}CD57⁺ T cells in patients with acute and stable conditions of CAD and relate the findings to CMV seropositivity.

Hypothesis

Since the aim of paper I was to establish the total and the methodological variation of major lymphocyte populations, there is no defined hypothesis.

The hypothesis in paper II was that LN analyses would reveal changes in among lymphocyte populations that are not detectable in blood.

The hypothesis in paper III was that decreased levels of NK cells in CAD patients compared to healthy controls are not permanent but can be restored over a 12-month period. A second hypothesis was that the NK cells differentiation markers and cytokine profile differ in ACS and SA patients.

The hypothesis in paper IV was that the prescence of CD28^{null} and CD57⁺ CD4⁺ and CD8⁺ T cells are higher in patients with CAD compared with controls, and that this phenomenon to some extent is associated with CMV infection.

Material and Methods

Subjects

In all studies, all blood samples were drawn between 8 and 12 a.m. For patients in paper III and IV, samples were always drawn immediately prior to coronary angiography (in non-STEMI patients within 24 h from admission). Different lymphocyte populations in blood or LN were prepared and analysed the same day as they were sampled. Cytokines and CMV were analysed in plasma obtained from ethylenediamine tetraacetic acid (EDTA) tubes and frozen within 1 hour in -20°C for analysis on a later occasion. For stimulation assays, blood was stimulated at the sampling day, frozen in -70°C (maximum 3 months) and after thawing further prepared and analysed.

Subjects included in the different studies are listed in Table I.

Healthy subjects did not use any medication and in case of current illness including ongoing infection the sampling was postponed to a later disease-free time point.

Inclusion criteria for SA subjects were in accordance with the Canadian Cardiovascular Society Classification classes II or III:

Class I: Angina only during strenuous or prolonged physical activity.

Class II: Slight limitation, with angina only during vigorous physical activity.

Class III: Moderate limitation, with symptoms with everyday living activities.

Class IV: Severe limitation with inability to perform any activity without angina or angina at rest.

Inclusion criteria for non-STEMI subjects were if electrocardiogram (ECG) changes included ST-T segment depression and/or T-wave inversion, and elevated troponins.

Exclusion criteria in study II were severe heart failure, renal or hepatic disease, neoplastic disease, immunologic disorders or treatment with immunosuppressive agents.

Exclusion criteria in study III and IV were severe heart failure or severe left ventricular systolic dysfunction, neoplastic disease, evidence of acute or recent (<2 months) infection, recent major trauma, surgery or revascularization procedure, immunologic disorders, treatment with immunosuppressive or anti-inflammatory agents (except low-dose aspirin).

Table I. Overview of subjects included in the studies.

Diagnosis	Paper I Longitudinal and methodological study	Paper II Cross-sectional study in blood and LN	Paper III Longitudinal study	Paper IV Longitudinal study
Non-STEMI (n)		13	31*	31*
Age		66 (49-82)	69 (50-83)	69 (50-83)
Male/Female (%)		77/23	74/26	74/26
SA (n)		13	34*	34*
Age		74 (61-84)	63 (44-77)	63 (44-77)
Male/Female (%)		77/23	82/18	82/18
Matched controls (n)		26**	37*	37*
Age		68 (49-77)	63 (45-77)	63 (45-77)
Male/Female (%)		77/23	76/24	76/24
Healthy controls (n)	15		7	
Age	42 (29-54)		42 (33-55)	
Male/Female (%)	27/73		29/71	
Total nr of patients and controls ***	15	52	109	102
Total nr of samples	493	52	418	190

** All patients and matched controls in paper III were also included in paper IV. ** Matched controls in paper II are also included in paper III and IV. *** In total 91 different patients and 59 healthy controls were included and investigated in the studies of this thesis (paper I-IV). Values are given as median (range).*

Paper I

For estimating biological variation in healthy individuals, blood was collected during 1997-2004. All persons did not participate during the whole period, but all participated at least 2 years (mean 4 years, range 2 – 7 years). Samples were taken approximately each month for each person. Demographic data of the individuals are listed in Table II and numbers of total observations for each lymphocyte population are listed in Table III. For valuation of methodological variation intra- and inter-assay calculations were done in separate experiments.

Table II. Distribution of sex, age at inclusion, duration of participation and number of sample occasions.

Number of subjects	Sex	Age at start of study	Total years in the study	Total sample occasions
11	female	40 (29-54)	4 (2-8)	30 (10-53)
4	male	43 (34-46)	4 (2-7)	34 (8-71)

Values are given as median (range).

Table III. Number of samples for each lymphocyte population.

CD	Lymphocyte population	Total samples	Total number of samples / month		
			Median	Min	Max
CD3	T cells	493	43	30	46
CD4	T-helper cells	493	43	30	46
CD8	Cytolytic T cells	493	43	30	46
CD19	B cells	302	27	18	30
CD16/56	NK cells	230	20	16	22

CD3, CD4 and CD8 were included in this study year 1997 whereas CD19 and CD16/56 were included in this study in year 2000 and 2001, respectively, explaining the lower number of observations.

Paper II

During 2008-2010 samples from LN and blood were collected from patients with SA ACS. blood was collected from a reference control group randomly selected from the population register and matched for gender and age. The controls were included mainly during the same time period as the controls.

Paper III and IV

During 2006-2010 blood was collected from SA and non-STEMI patients that were followed longitudinally over 12 months. Samples were taken at day 1 after admission (always prior to coronary revascularization) and at 3 and 12 months. All persons did not participate during the whole period. Controls matched for gender and age were sampled at one occasion. Patients and control persons were included continuously during the sampling period. In one series of experiments, blood samples were taken for *ex vivo* assays. For control of longitudinal variation, healthy subjects were followed during 2007-2011 (Table IV). Demographic data of the individuals are listed in Table IV and numbers of total observations for each lymphocyte population were 228.

Table IV. Distribution of sex, age at inclusion, duration of participation and number of sample occasions.

Number of subjects	Sex	Age at start of study	Total years in the study	Total samples
5	female	40 (35-47)	5 (5-5)	35 (12-51)
2	male	48 (40-55)	5 (5-5)	27 (27-27)

Values are given as mean (range).

Ethical considerations

Study I was approved by the Regional Ethics Committee of Linköping University. Study II-IV was conducted in accordance with the ethical guidelines of Declaration of Helsinki, and approved by the Ethical Review Board of Linköping University. Written informed consent was obtained from all study participants (paper I-IV).

Laboratory techniques

Flow cytometry was used for analysis of lymphocyte phenotypes in peripheral blood in paper I-IV and in paper II also in lymph nodes. Flow cytometry was also used for detection of intracellular cytokines in paper IV. Enzyme-linked immunosorbent assay (ELISA) was used for detection of soluble cytokines in paper III and IV and for CMV in paper IV.

Flow cytometry

Flow cytometry is a process of performing measurements on cells or particles that are in a liquid suspension. It is a technique that simultaneously measures and analyses multiple characteristics of single cells. By hydrodynamic focusing of the sample stream, the cells are lined up in a single-cell stream. As the cells pass through the laser beams, fluorochromes are excited to a higher energy state, followed by emitting light of a longer wavelength. The emitted light and the scattered light is detected by photomultiplier (PMT) tubes and then converted into electrical pulses and further processed for digitalizing data (Figure 6). Based on light scatter (forward and side scatter) and fluorescence it is possible to determine different kinds of cells or particles stained with antibodies conjugated with fluorochromes in blood samples. Side-scattered light (SSC) is proportional to the internal complexity of the cell whereas forward-scattered light (FSC) is proportional to the size of the cell. The amount of the fluorescence signal is proportional to the number of fluorochrome molecules on the cell. When using multicolour analysis, emitted wavelengths from one fluorochrome can despite appropriate filters spill over to a channel for another fluorochrome. This can be avoided by manually or automatically performed compensation of the fluorochromes.

A list of monoclonal antibodies and other staining reagents used in this thesis is presented in Table V.

Figure 6. Schematic principle of flow cytometry. When cells pass through laser beams, light is scattered and emitted, which reveals information about the cells' size (FSC), complexity (SSC) and cell type.

Table V. Monoclonal antibodies against cell surface and intra-cellular markers used in flow cytometry analysis.

Marker	Mainly expressed by / function	Clone	Fluorochrome	Used in paper
CD3	T cells	SK7	FITC	I, II, III, IV
CD3	T cells	SK7	PerCP	II, III, IV
CD3	T cells	SK7	APC-H7	II
CD3	T cells	UCHT1	PB	II
CD4	T helper cells	SK3	PE	I, IV
CD4	T helper cells	SK3	PerCP	II
CD4	T helper cells	SK3	PE-Cy7	II, IV
CD4	T helper cells	SK3	APC	III, IV
CD8	T cytotoxic cells	SK1	PE	I
CD8	T cytotoxic cells	SK1	APC-H7	II, IV
CD8	T cytotoxic cells	SK1	APC-Cy7	II, IV
CD19	B cells	J25C1	PE	I
CD19	B cells	J25C1	APC	II, III
CD25	Activated T and Treg cells, part of the IL-2R complex	2A3	APC	II
CD28	Co stimulatory, T cell activating	L293	PE	IV
CD45	Leukocytes	2DI	PerCP	I, II, III, IV
CD16/56	NK cells	B73.1/NCAM 16.2	PE	I, III
CD56	NK cells	NCAM16.2	PE	II
CD56	NK cells	NCAM16.2	PE-Cy7	II, III
CD56	NK cells	NCAM16.2	APC	II, III
CD56	NK cells	B159	HV450	III
CD57	NK differentiation	HNK-1	FITC	III, IV
CD69	Early activation	FN50	PE-Cy7	II, III, IV
CD69	Early activation	L78	PE	III, IV

Table V continued. Monoclonal antibodies against cell surface and intra-cellular markers used in flow cytometry analysis.

Marker	Mainly expressed by / function	Clone	Fluorochrome	Used in paper
CD94	NK inhibitor of cytolytic activity	HP-3D9	APC	III
HLA-DR	Late activation of T cells, peptide presentation on APC	L243	FITC	II, III, IV
CCR4 (CD194)	Chemokine receptor. Activation, Th2 associated	1G1	PE-Cy7	II
IL-18r	Activation, Th1 associated	70625	PE	II
Foxp3	Transcription factor for regulatory CD4 T cells	PCH101	PE	II
KIR (NKB1)	NK inhibitor of cytolytic activity	DX9	FITC	III
KIR (NKAT-2)	NK inhibitor of cytolytic activity	DX27	PE	III
NKG2a (CD159a)	NK inhibitor of cytolytic activity	Z199	PE	III
IFN- γ	Th1 associated	257.11	PE	IV
TNF	Pro-inflammatory	6401.1111	PE	IV
IL-10	Anti-inflammatory	JES3-9D7	PE	IV
IL-13	Th2 associated	JES10-5A2	PE	IV
IL-17	Th17 associated	SCPL1362	PE	IV

3-color analysis (paper I)

In paper I, flow cytometry was performed by 3-color on a FACSCalibur (BD Biosciences, San Jose', CA) equipped with an argon laser 488 nm. Data analyses were performed with Attractors™ (BD Biosciences) or Cellquest™ software. Control of the instrument settings was done daily with CaliBRITE™3 Beads (BD Biosciences) and FACSCComp™ software according to the standard protocol. Manual compensations were done. In this paper both methodological and biological variation was determined for T cells, T helper cells, T cytotoxic cells, B cells and NK cells. The methodological variation included both inter-and intra-assay. Acquisition of samples was done until 5000 lymphocytes were detected.

Gating strategies

To decide the number of lymphocyte, a BD Trucount™ tube (BD Biosciences), containing an exact number of fluorescent lyophilized beads, was used. In the same tube, numbers and proportions of lymphocyte populations were assessed based on CD45⁺ and side-scatter characteristics. Thereafter, T cell populations were determined by a gate based on CD3 expression whereas B cells and NK cells were based on a CD3⁻ gate.

4-7 color analysis (paper II-IV)

In paper II-IV, flow cytometry was performed by 4-7-color analysis on FACSCanto A or FACSCanto II (BD Biosciences) equipped with 3 lasers; a blue 488 nm, a red 633 nm and a violet 405 nm. Data analyses were performed with FACSDiva™ 6.1.2 software (BD Biosciences). Control of the instrument settings was done daily with 7-color Setup Beads™ with FACSDiva software or Cytometer Setup and Tracking beads™ (BD Biosciences) with Cytometer Setup and Tracking™ software according to the standard protocol. Automatic compensations were done using Comp beads™ (BD Biosciences) for each new tandem-conjugate and at least every 3 months for all antibodies.

Gating strategies

To decide the number of lymphocytes in peripheral blood, a BD Trucount™ tube (BD Biosciences), containing an exact number of fluorescent lyophilized beads, was used in the same way as in paper I. The absolute number of cells from this tube was then applied in all other tubes. To define discrete or continuously expressed markers, negative populations were used to define the cut-off between positive and negative populations. For paper II, a median of 23 500 (range 6 200 – 90 300) peripheral lymphocytes and 95 000 (range 4 800 – 116 700) lymph node cells were analysed. For paper III-IV acquisition was done until 10 000 lymphocytes were detected both for lymphocyte subset and cytokine-positive cell analysis. An example of gating strategy of lymphocyte subpopulations with 6-color analysis is shown in Figure 7.

Figure 7. Example of gating strategy with 6-color analysis. Paper III and IV; lymphocyte subpopulations were assessed based on forward and side-scatter characteristics (upper left). Thereafter T cells were determined by a gate based on CD3 expression (upper centre). Paper III; NK cell bright and dim were gated based on CD3-negative cells and the expression of CD56 (upper right). Thereafter the CD56^{dim} population was divided into CD57⁻ and CD57⁺ (middle right). Paper IV; T helper cells were gated based on CD3 and CD4 expression (middle left) and T cytotoxic cells were gated based on CD3 and CD8 expression (middle centre). Finally the CD4⁺ population was divided into CD4⁺28^{null} and CD4⁺28^{null}57⁺ (lower left) and the CD8⁺ population divided into CD8⁺28^{null} and CD8⁺28^{null}57⁺ (lower right).

Fluorescence minus one (FMO)

Traditionally isotype controls are used in order to identify non-specific binding and gating thresholds. Since the boundaries for positivity also may depend on the amount of fluorescence in all channels, isotype controls are not appropriate in multicolor experiments. Instead of isotype controls FMO gating strategy was used in the experiment setup to identify weakly expressed cells and discriminate the population from the negative population. The FMO combines all the fluorochromes in a panel except for the one that is being measured (Figure 8).

Figure 8. FMO gating strategy. To appropriately distinguish populations with weak expression of fluorescence, FMO gating strategy can be done. In the first plot on the left cells are unstained. In the second plot all antibodies are present except FITC and the threshold is defined for the FITC channel. In the third plot all antibodies are present except PE and the threshold is defined for the PE channel. In the last plot on the right all antibodies are combined and the thresholds for positive cells is defined from previous FMO plots.

Quality

During the time period for study I – IV the laboratory participated in at least 2 external quality programs for immune phenotyping (UK-Nequas 1997 - 2004 and Equalis 2001 - 2011). Since 1996, the laboratory is accredited according to SWEDAC ISO/IEC 17025 standards.

Validating FACS Calibur vs FACSCanto II

In paper I flow cytometry analysis was performed on a FACSCalibur whereas flow cytometry analysis in paper II was performed on FACSCanto II and in paper III-IV on FACSCanto A and II. In paper II-IV reversed pipette technique was used.

To interpret results from FACSCalibur and results from FACSCanto A and II validating analyses were performed to compare the instruments. For further description see “Results and Discussion”.

ELISA (paper III and IV)

ELISA is a technique used for detection or quantification of an antibody or an antigen in a liquid. Different kinds of ELISA are used depending on the sample constitution and what substance that will be measured. A brief description of different ELISAs follows.

In an **Indirect ELISA** a microtitre plate is pre-coated with antigen and then samples that are to be examined for presence of antibodies are added to the well. After incubation, a second antibody, directed against the isotype of antibody to be detected, is added. This secondary (or detection) antibody is conjugated with an enzyme, and after incubation a substrate for this enzyme is added. Between all steps unbound antibody / antigen is removed by repeated washings. The substrate is without colour, while the product has a colour and therefore the amount of antibodies present in the sample is proportional to the colour intensity. The optical density is determined by a spectrophotometer.

In a **Sandwich ELISA** the microtitre plate is coated with a capture antibody and then the sample containing the antigen is added, followed by adding of the secondary (detection) antibody, which binds to another epitope of the antigen. Between all steps unbound antibody / antigen is removed by repeated washings. Finally substrate is added and converted by the enzyme into a coloured product or chemiluminescent signal.

In paper III and IV a sandwich chemiluminiscent enzyme-linked immunosorbent assay (QuantiGlo) Chemiluminiscent Elisa, R&D systems was used for detecting the production of IL-6 and IL-15 with a detection limit of 0.48 pg/ml for IL-6 and 1.03 pg/ml for IL-15. The principle for this ELISA is that antibodies against the cytokines are pre-coated. Samples or standards are added and cytokines that are present will bind to the immobilized antibody. Unbound materials are washed away. In the next step a second HRP-labelled antibody (detection antibody) is added and it binds another (empty) epitope of the cytokine. Unbound detection antibody is washed away. Finally substrate is added and light is produced in proportion to the amount of cytokines in the sample. For measuring the intensity of the emitted light, a microplate luminometer is used (Figure 9).

Figure 9. Principle of a sandwich ELISA used in paper III and IV for detection of IL-6 and IL-15. The wells of a microtitre plate are pre-coated with antibody against the cytokine of interest. The sample containing cytokines (antigen) is added. Finally a secondary labelled antibody is added.

In paper III and IV a two-step sandwich chemiluminescent microparticle immunoassay (CMIA) (Architect, Abbott Laboratories, Abbott Park, IL, USA) was used for detection of IgG and IgM antibodies against CMV. Antibody levels ≥ 6.0 IU/ml were considered positive for IgG. For IgM antibody levels < 0.85 IU/ml were considered negative, $0.85 - 1.0$ IU/ml borderline and ≥ 1.0 IU/ml positive. In the first step, sample, assay diluent, and coated paramagnetic microparticles are combined. After washings, conjugate is added to create a reaction mixture. After another wash cycle, pre-trigger and trigger solutions are added to the reaction mixture. The resulting chemiluminescent reaction is measured as relative light units.

Intracellular cytokine staining (paper III and IV)

FastImmune™ Cytokine Flow Cytometry is a flow cytometric method that allows the detection of functional populations of lymphocytes that respond to specific antigens.

The details of the method are described in paper III and IV. The principle is that whole blood is stimulated with mitogen (phorbol myristate acetate (PMA)/Ionomycin) or antigen (a CMV peptide mix) together with costimulatory antibodies against CD28/CD49d in the presence of the protein secretion inhibitor Brefeldin A. EDTA solution is added in order to stop activation and to remove adherent cells. Cells are then lysed and fixated and stored at -70°C . At the time of staining for cytokines and cell surface molecules, the frozen cells are thawed and permeabilized. Anti-cytokine and cell surface antibodies are finally added before flow cytometry analysis is performed.

Statistics

The statistical guidance resource at Linköping University was consulted for the statistical analyses in paper I.

All statistical analyses were performed using statistical package for the social sciences (SPSS)[®] version 11.5 (paper I), 17.5 (paper II) and 19 (paper III-IV) software (SPSS Inc., Chicago, IL, USA). Mainly non-parametric methods were performed in paper II-IV since several of the flow-cytometry data were not normally distributed.

Statistical significances were set at a two-tailed p-value of < 0.05 (paper II-IV).

Different statistical methods used in this thesis are listed in Table VI.

Table VI. Statistical methods used in paper I-IV

Method	Description	Paper
χ^2 test	Distribution of nominal data	III, IV
Coefficient of variation (CV) % (SD / mean) x 100	Methodological intra-, inter-assay variation and biological longitudinal study	I
Two-way ANOVA	Parametrical test investigating two explaining variables with multiple observations in the longitudinal study	I
Kruskal-Wallis test	Non-parametric test for comparisons between several groups (ACS, SA and controls)	III, IV
Mann-Whitney <i>U</i> -test	Non-parametrical test for comparisons between two groups (performed if Kruskal-Wallis test showed a tendency defined as $p < 0.1$)	II, III, IV
Friedman's test	Non-parametrical test comparing several paired data within groups (ACS or SA patients)	IV
Wilcoxon's signed-ranks test	Non-parametrical test for pair-wise comparisons within ACS or SA patients	II, III, IV
Spearman's rank correlation test	Non-parametrical correlation analyses	II, III

Results and Discussion

In this thesis, lymphocyte populations, cytokine secreting cells, soluble cytokines and antibodies against CMV were investigated in order to assess differences between SA, non-STEMI patients and healthy individuals, at inclusion in the study as well as during longitudinal follow up.

To assure the quality of the flow cytometry analyses, as well as for the longitudinal studies, both the methodological variation and the biological variation over time were estimated.

Methodological aspects

Since the study in paper I was performed on a FACSCalibur instrument with 3-color analyses and Attractors software, whereas the studies in paper II – IV were performed on FACSCanto instruments with at least 4-color combinations and Cellquest or FACSDiva software, comparative analyses were performed as validation. An overview of different validation analyses concerning $CD3^+$, $CD3^+4^+$, $CD3^+8^+$, $CD3^+19^+$ and $CD3^-16/56^+$ are listed in Table VII and correlation plots are shown in Figure 10. The results showed over all good correlations meaning that results from different instruments and protocols in this thesis are comparable and no correction calculations were considered necessary.

Table VII. Comparative analyses between different protocols and instruments.

Number of samples	FACSCalibur	FACSCalibur	FACSCanto “old”	FACSCanto “new”
9	3-color Attractors	3-color Cellquest		
8	3-color Cellquest	4-color Cellquest		
5		4-color Cellquest	4-color FACSDiva	
10			4-color FACSDiva	4-color FACSDiva

Figure 10. Results from 4 different comparisons of the major lymphocyte subpopulations. Upper left; comparison of Attractors software vs Cellquest software with 3-color combinations gave a result of $R^2 = 0.9985$. Upper right; comparison of 3-color combinations vs 4-color combinations on FACSCalibur gave a result of $R^2 = 0.9904$. Lower left; comparison of 4-color combinations on FACSCalibur vs FACSCanto II gave a result of $R^2 = 0.9966$. Lower right; comparison of 4-color combinations on an “old” FACSCanto II vs a “new” FACSCanto II gave a result of $R^2 = 0.9988$. All dot plots show the correlations of $CD3^+$, $CD3^+4^+$, $CD3^+8^+$, $CD3^+19^+$ and $CD3^+16/56^+$ populations, hence covering a large range of percentages.

Methodological studies in paper I

When measuring both clinical and research samples it is important to be familiar with the methodological variation. In flow cytometry there are several critical parameters that influence the variation within a laboratory; in principal related to instrument variation, variations in the preparation procedure and variations in the gating performance. The more persons involved, the more variation of the final result. To assess aspects of methodological variation intra- and inter-assay enumerations were done. In this paper we determined the variation of proportions and numbers of T cells, T helper cells, T cytotoxic cells, B cells, NK cells and T helper/T cytotoxic ratios based on 3-color combinations on a FACSCalibur instrument. The samples were obtained as a part of a control program in the accreditation procedure.

For intra-assay determination, one sample was prepared once and thereafter acquired directly 10 times after each other on the same flow cytometer and by the same technician. The procedure was repeated once with another sample. The results of the intra-assay experiment, reflecting the variation of the instrument, showed a CV ranging from 1 to 7 % for counts and 0 - 6 % for proportions of lymphocyte populations (Table VIII).

Table VIII. Intra- and inter-assay variations as well as total variation over time, thus including methodological and biological variation (Paper I).

	Intra-assay CV (%)		Inter-assay CV (%)		Total CV (%)	
	Count (range)	% (range)	Count (range)	% (range)	Count (range)	% (range)
CD3 ⁺	2 (1-3)	1 (0-1)	3 (2-7)	1 (1-2)	15 (9-24)	4 (1-9)
CD3 ⁺ 4 ⁺	2 (2-3)	2 (1-2)	3 (1-7)	2 (1-3)	16 (10-28)	6 (5-10)
CD3 ⁺ 8 ⁺	3 (2-4)	2 (1-2)	4 (3-6)	3 (1-5)	18 (13-30)	9 (5-13)
CD3 ⁺ 19 ⁺	4 (3-6)	4 (0-6)	11 (5-25)	9 (6-18)	18 (12-25)	11 (8-19)
CD3 ⁺ 16/56 ⁺	6 (4-7)	5 (3-6)	8 (5-10)	7 (6-9)	25 (14-34)	22 (14-30)
CD4/CD8 ratio	4 (3-5)		3 (0-5)		12 (5-18)	

CV % was calculated from the mean of each lymphocyte population.

For inter-assay determination, 2 different samples were prepared and evaluated at 4 different time points during 24 hours. One sample was prepared twice at all time points by 5 experienced cytometry technicians, the other sample was prepared the same way by 4 of the technicians. In total 18 preparations were done and they were all analysed on one instrument by the same technician that prepared the sample. As expected, the results of this inter-assay determination including both instrument, preparation and gating variations, resulted in higher CVs than the intra-assay determination (Table VIII). B cells and NK cells showed the highest variation, probably in part because of their lower cell numbers, meaning that a small difference leads to high relative variation.

Longitudinal methodological studies

Paper I

In paper I data obtained in 1997 – 2004 from 15 healthy human adults enrolled in a control program, were retrospectively studied in order to evaluate the variation over time in healthy individuals. Samples were taken approximately each month and to minimize the biological variation the sampling time was between 8 a.m. and 12 a.m. Except from current illness including ongoing infection, one person was excluded because of pregnancies since the immune system changes during pregnancy

The total variation over time for different lymphocyte populations is shown in Table VIII. The total variation includes both methodological and biological variations. As expected, the total variation therefore exceeds the methodological variation. It was also expected with a higher variation of numbers than of proportions (%), since it is well known that assessing of numbers is more difficult and subject to higher variation in external quality programs in general. For T cell populations (CD3⁺, CD4⁺ and CD8⁺), the total variation was 4 – 9 % for proportions and

15 – 18 % for numbers, which is clearly higher than the inter-assay variation of 1 to 4 % (both for numbers and proportions). Still, considering that the total variation relates to changes over several years, the T cell populations can be regarded as stable. B cells and in particular NK cells showed a slightly higher total variation over time (numbers; 18 – 25 %), but for these populations also the inter-assay variation was higher (8 – 11 %). Taken together, although the total variation is higher than the methodological variation, it is clear that the methodological variation must be taken into account in the total variation. Furthermore, although the biological variation over time in general is larger than the methodological variation, the lymphocyte populations studied here are rather stable over several years.

To evaluate the longitudinal results in relation to time of year for sampling, a two-way ANOVA for unbalanced data was performed; first evaluating interactions between different individuals and secondly monthly variation. There were no inter-individual differences, while differences over the year were indicated. Therefore a post-hoc test was done to reveal between which months differences occurred. As shown in Figure 11 no significant differences were noted for proportions of lymphocyte populations whereas there was a tendency ($p < 0.1$) for absolute numbers of $CD3^+$ cells between October and June as well as between October and November. Further, there was a significant difference ($p < 0.05$) for absolute numbers of $CD8^+$ cells between October and June as well as between October and July for $CD3^+8^+$ ($p < 0.05$ for both comparisons, (Figure 12). Although several previous reports suggested variations over time, no consistent results were found. In fact there are several issues that might impact the outcome of the results, for instance some of the studies are based on either old techniques with microscopy (Bratescu and Teodorescu, 1981, Abo et al., 1985, Levi et al., 1988, Afoke et al., 1993) or 2-color analysis (Paglieroni and Holland, 1994) , which is not comparable with current multicolour techniques. Other factors that may affect the results is the sampling time (Ritchie et al., 1983, Levi et al., 1988, Pasqualetti et al., 1988, Fukuda et al., 1994, Northern et al., 1994, Mazzocchi et al., 1998), smoking (Schaberg et al., 1997) , exercise training (Schaberg et al., 1997) and immunological conditions (Matthiesen et al., 1996, Termorshuizen et al., 2002).

Although we considered these factors, we could not find any clear explanation for our findings that in particular October differed in absolute numbers of $CD3^+$ and $CD3^+8^+$ but speculated that infections or diminished daylight might influence the result. However, it is perhaps more likely that the differences, which were considered minor, appeared by coincidence.

The main results in this paper were that the methodological variation in general was low and that the total variation, which includes methodological and biological variation, was moderate. These figures are useful when assessing differences in longitudinal research studies as well as in the clinical laboratory when evaluating repeated measurements in patients. Further, there were no major variations over the year for different lymphocyte populations. Thus, time of the year should not be necessary to take into consideration in repeated measurements of major lymphocyte populations, neither in research nor in the clinic. A prerequisite is that procedures are standardized with regard to sampling, preparation and analysing data.

Figure 11. Proportions of lymphocyte populations in relation to month (paper I). No significant differences ($p < 0.1$) were noted over time. Bars denote means and whiskers show 1 SD. The months are defined from 1 (January) to 12 (December).

Figure 12. Absolute counts for lymphocyte populations in relation to month (paper I). A tendency was noted for $CD3^+$ ($*$) = $p < 0.1$ for October (10) compared with June (6) and November (11), respectively. Absolute count $CD8^+$ was significant ($*$) = $p < 0.05$ lower in October compared with June (6) and July (7), respectively. Bars noted as 1 SD. The months are defined from 1 (January) to 12 (December).

Paper III

In paper III we tested the longitudinal variation for major lymphocyte subsets during the 5-year study time with the currently used instrument, settings and protocols. In total 228 samples from 7 healthy controls were tested. The total variations over time for numbers and proportions of lymphocytes were determined by calculating mean of CV % for each lymphocyte population of each individual in the same way as in paper I. For proportions the CV % varied from mean 3 (range 2-4) % for CD3⁺ cells to 20 (18-22) % for NK cells (Table IV). These results are similar to the findings in paper I (Table VIII). The results confirm the findings in paper I and show that they are valid also on another instrument using a different protocol. The findings are also useful when evaluating the variation of NK cells over time in paper III.

Table IV. Total variation of healthy controls during 5 years (Paper III).

	Mean CV (%)	
	Count (range)	% (range)
CD3 ⁺	15 11-21	3 2-4
CD3 ⁺ 4 ⁺	15 11-20	6 5-8
CD3 ⁺ 8 ⁺	16 13-24	6 3-9
CD3 ⁻ 19 ⁺	18 13-26	11 8-19
CD3 ⁻ 16/56 ⁺	26 23-31	20 18-22
CD4/CD8 ratio	11 7-15	

Major lymphocyte populations in lymph node vs blood (paper II)

As expected, comparing blood to LN showed several marked differences. The pattern of different lymphocyte populations in lymph nodes was the same for SA and non-STEMI patients. As shown in Figure 13 and Table X the proportions of CD3⁺, CD8⁺ and NK cells were higher in blood than in LN in both patient groups, whereas B cells were highly enriched in LN. The proportions of CD4⁺ T cells were similar in blood and LN. In blood the CD56^{bright} fraction was rare, whereas in lymph node it constituted a larger population. This is in line with a previous report that also demonstrated a link between adaptive and innate lymphocytes where CD56^{bright} cells were enriched in LN and stimulated by T cell-derived IL-2 to secrete IFN- γ (Fehniger et al., 2003). It is also in line with the concept that CD56^{bright} cells are regulatory cytokine secreting cells rather than effector cytotoxic NK cells (which is the CD56^{dim} population) (Poli et al., 2009).

Previous studies mostly looked at LN from different cancer patients, although the examined LNs were free of disease. Regarding major lymphocyte populations they got similar results for lymph nodes as in our study (Morton et al., 1986, Takenoyama et al., 1996, Santin et al., 2001, Vidal-Rubio et al., 2001, Battaglia et al., 2003). In our study we evaluated but found no differences in LN cells between acute and stable conditions of CAD. We can not exclude that the results would be different if draining (upper mediastinal) lymph nodes were used instead of the internal mammary artery lymph nodes that we used. Results of subpopulations will follow.

Figure 13. Schematic overview of distribution of major lymphocyte populations and some subpopulations in lymph node and blood. Representative examples from one SA patient. The pattern was the same for SA and non-STEMI patients.

Major lymphocyte populations in peripheral blood (paper II-IV)

In blood the major findings in paper II (Table X) were significantly lower proportions of NK cells in non-STEMI patients compared with SA and controls ($p < 0.05$ for both comparisons), and in parallel, $CD3^+$ T cells in total were significantly increased in non-STEMI compared to controls ($p < 0.05$), which seemed to depend on an increase in the $CD8^+$ population ($p < 0.1$). As shown in Table X the proportions of NK cells were reduced in both SA and non-STEMI patients compared to controls in paper III/IV. In line with the reduction of NK cells the proportions of $CD3^+$ T cells were increased, here depending on increase in the $CD4^+$ T cells.

Thus when comparing paper II and paper III/IV results consistently shows that NK cells are lower in CAD patients compared with controls, which is also in line with previous studies from our group and others (Jonasson et al., 2005, Hak et al., 2007, Backteman et al., 2012, Hou et al., 2012). However some differences were noted across papers. While NK cells were consistently decreased in non-STEMI patients, they were decreased in SA patients as well in paper III/IV but not in paper II. Another discrepant finding was that $CD4^+$ T cells were increased in both the non-STEMI and the SA group in paper III/IV, while they were not increased in CAD patients in paper II. The findings in paper III/IV are in line with our

previous findings (Jonasson et al., 2002, Jonasson et al., 2005). The main reasons for the discrepancies between paper II and paper III/IV are different time point of sampling and clinical presentation. In paper II the sampling time of non-STEMI patients was approximately 14 days after ACS and in paper III/IV within 24 h from admission, thus at different time points during the process. One consequence of the different time points is that more non-STEMI patients in paper II were treated with statins, which may affect lymphocyte populations and Th1 responses already after a few days (Link et al., 2006). Furthermore the SA patients in paper II were a more homogenous group regarding CAD severity and might also have been in a phase with optimized anti-anginal medication in comparison with the SA group in paper III/IV.

Taken together, the studies clearly show that there is an aberration in NK cells in CAD (to be further discussed regarding subpopulations and longitudinal findings below). It is also possible that the selection of material in terms of disease state, time of sampling and treatment has substantial impact on findings.

Table X. Distribution of major lymphocyte subpopulations in paper II-IV.

		Paper II			Paper III/IV		
		Non-STEMI (=13)	SA (=13)	Controls (=26)	Non-STEMI (=31)	SA (=34)	Controls (=37)
CD3⁺	Blood	72 (66-79) ^A	67 (64-72)	64 (58-72)	76 (68-80) ^B	75 (71-80) ^B	65 (60-79)
	LN	55 (45-60)**	60 (48-67) ^(*)				
CD3⁺4⁺	Blood	42 (35-46)	40 (27-51)	39 (32-47)	49 (42-56) ^B	49 (42-54) ^A	39 (33-49)
	LN	41 (37-47)	43 (35-53)				
CD3⁺8⁺	Blood	29 (20-34) ^C	24 (17-35)	21 (18-28)	25 (19-34)	24 (19-31)	23 (19-29)
	LN	14 (10-17)**	15 (9.3-21)*				
CD3⁺19⁺	Blood	11 (6.9-13)	7.0 (5.6-11)	9.6 (7.0-14)	8.7 (7.8-12)	11 (6.8-12)	9.6 (7.5-14)
	LN	43 (38-53)**	39 (30-50)**				
CD3⁺56⁺	Blood	12 (9.4-19) ^{A, D}	20 (12-23)	21 (13-31)	12 (9.8-15) ^B	12 (9.1-18) ^A	18 (11-29)
	LN	1.5 (1.3-2.4)**	1.6 (1.2-2.0)**				

Values are given as median (interquartile range).

Values ^A $p < 0.05$, ^B $p < 0.01$, ^C $p < 0.1$ refer to differences compared with controls. Values ^D $p < 0.05$ refer to differences compared with SA.

Values ^(*) $p = 0.07$, * $p < 0.05$, ** $p < 0.01$ refer to differences between LN and blood.

Significant differences between patient groups and healthy subjects are highlighted.

Lymphocyte subpopulations (paper II)

Lymphocyte subpopulations in paper II were assessed by markers of activation (CD69, HLA-DR) and regulatory markers for type 1 (Th1-associated) responses (IL-18R), (Chan et al., 2001), type 2 (Th2-associated) responses (CCR4), (Nagira et al., 1998) and Treg cells (based on expression of CD4, CD25 and Foxp3, see below). In general, findings were similar in SA and ACS patients and therefore data are mainly discussed in the perspective of LN *versus* blood.

As shown in Table XI and Figure 13, CD4⁺ T cells expressing the early activation marker CD69⁺ were few in blood but numerous in LN. This is in line with a previous study demonstrating the enrichment of CD69⁺ lymphocytes in both tumour and control LNs (Vidal-Rubio et al., 2001). Regarding the late activation marker HLA-DR, we found that its expression on CD4⁺ and CD8⁺ cells was similar in blood and lymph nodes. Thus, there is a discrepancy of activation markers. Traditionally, CD69 was regarded simply as a marker of early activation, being expressed on the cell surface of T cells hours after activation (Hara et al., 1986, Lopez-Cabrera et al., 1993).

However, recent data suggest that CD69 is an immunoregulatory molecule (Martin et al., 2010), involved in regulation and limitation of Th1, Th2 as well as Th17-mediated inflammation. Also, CD69 has recently been shown to be critically involved in the egress of T cells from LNs by binding the sphingosine 1-phosphate receptor 1 (S1PR1), which controls the migration of lymphocytes from LNs (Swan et al., 2012).

Therefore, it is likely that the profound enrichment of CD69⁺ CD4⁺ cells in lymph nodes reflect immunoregulatory functions and migratory properties of these cells, rather than a state of activation, which is in line with the finding that HLA-DR expressing CD4⁺ cells were not increased in LNs as compared with blood.

The alternative role of CD69 (beyond marker of activation) is also supported by our findings of abundant coexpression of Foxp3 and CD69 in lymph node Treg cells, but not in peripheral blood Treg cells. In blood only about 3-6 % of Foxp3⁺ Tregs expressed CD69 whereas approximately 45-65% of Foxp3⁺ Tregs expressed CD69 in LN. A role for CD69 in identifying a suppressive Treg population has recently been proposed (Cortes et al., 2014).

In atherosclerosis Treg cells are suggested to have an atheroprotective role through suppressive actions on Th1 polarization, and loss of this population might result in plaque destabilization and rupture. Although previous studies (Mor et al., 2006, Han et al., 2007, Ammirati et al., 2010) have shown reduced numbers of Treg cells in blood in ACS compared with SA patients, we were not able to repeat these findings, neither in blood nor in LN.

Depending on the definition and gating strategy for Tregs, the proportions differed between LN and blood (Figure 14). With the (old) classical way of defining Tregs by CD4^{dim}25^{bright} (Mjosberg et al., 2009) there were no significant differences neither between blood and LN nor between SA and ACS patients. When Foxp3 was included as a marker for Tregs, the proportions were almost twice as big in LN, but still without differences between SA and ACS patients (Table XI).

Figure 14. Gating strategies for regulatory T cells (Treg) in lymph node. T cells were defined by their CD3 expression (panel A) and then transferred for evaluation of Treg markers. Panel B shows CD4^{dim}/CD25^{bright} cells, defined by their slightly lower (dim) CD4 expression and high (bright) expression of CD25. Panel C shows Foxp3 expressing CD4 cells using the CD8 Foxp3 expression as cut-off. Panel D shows CD4⁺ cells with high (bright) expression of CD25, slightly lower (dim) CD4 expression in combination with a high Foxp3 expression. Values are given as percent of CD3⁺4⁺ cells. Examples from lymph node show dot plots representative for both SA and ACS patients.

The identification of Treg cells has improved a lot and it is also evident that Tregs are not a homogeneous population. Despite that several markers have been suggested, there is no perfect way to define Treg cells by cell markers. One problem is that both CD25 and Foxp3 are upregulated by activation. One way to circumvent this problem was to define Treg cells as CD4^{dim}/CD25^{bright}, which led to an enrichment of Foxp3^{bright} and CD127^{low} cells (Mjosberg et al., 2009). Another way to address the issue of activation is to use CD45RA as a marker of naïve or resting Treg cells, thereby dismissing CD45RA-negative/Foxp3 expressing cells as non-suppressive cells (Miyara and Sakaguchi, 2011). This strategy was not known at the time of our study. Recent data from the group (Hasib et al., data to be published), shows that both ACS and SA patients differ from controls regarding Treg phenotype (when including CD45RA), as well as Treg function. In our study we did not find any differences in Treg frequencies between CAD patients and controls. This discrepancy to other studies (Mor et al., 2006, Han et al., 2007, Ammirati et al., 2010) may be due to the markers that were used, but also to the heterogeneity in CAD patient populations.

Table XI. Distribution of T cell activation markers and regulatory T cells in blood and LN respectively.

		Non-STEMI (=13)	SA (=13)	P
CD3⁺4⁺69⁺ % of CD4⁺ Early activation	Blood	1.0 (0.9-1.8)	1.2 (0.9-1.8)	NS
	LN	49 (41-58)**	40 (38-50)**	NS
CD3⁺4⁺HLA/DR⁺ % of CD4⁺ Late activation	Blood	10 (7.5-13)	14 (7.4-18)	NS
	LN	13 (9.9-16)	13 (10-17)	NS
CD3⁺8⁺HLA/DR⁺ % of CD8⁺ Late activation	Blood	32 (17-60)	37 (28-43)	NS
	LN	35 (22-54)	32 (25-49)	NS
CD3⁺4^{dim}25^{bright} % of CD4⁺ Treg	Blood	3.0 (2.2-4.7)	2.9 (2.4-4.8)	NS
	LN	3.8 (2.0-8.0)	4.9 (3.7-6.6)	NS
CD3⁺4^{dim}25^{bright} Foxp3⁺ % of CD4⁺ Treg	Blood	2.1 (1.3-2.8)	2.0 (1.5-3.0)	NS
	LN	3.6 (2.1-6.3)**	4.5 (2.8-5.3)**	NS
CD3⁺4⁺ Foxp3⁺ % of CD4⁺ Treg	Blood	4.5 (3.0-5.1)	5.2 (3.1-5.6)	NS
	LN	17 (11-19)**	15 (10-17)**	NS

Values are given as median (interquartile range). No significant (NS) differences ($p > 0.05$) were noted between SA and non-STEMI patients. Comparing blood and lymph nodes, there were significant differences in CD3⁺4⁺69⁺ and some Treg populations. Values ** $p < 0.01$ refer to differences between LN and blood.

IL-18R (a member of the Toll-IL-1 receptor family) has been demonstrated (Chan et al., 2001) to be a stable cell surface markers for human type 1 lymphocytes including CD4⁺, CD8⁺ and NK cells. In our study we used IL-18R as a marker for type 1 cells. CCR4 was used

as a proxy for type 2 cells since is the receptor for Th2-recruiting chemokines (Nagira et al., 1998). The proportions of CCR4 on CD4⁺ cells were more frequent in blood compared to LN, without any differences between SA and ACS patients (Table XII). IL-18R expressing CD4⁺ cells were also more frequent in blood compared to LN (statistically significant for ACS but not for SA patients. Possibly, these differences between blood and LN may reflect that most CD4⁺ cells in the LN have not encountered an antigen and have not been polarized, thus explaining the lower frequency of cells with polarization markers in the LNs. For NK cells, the proportions of IL-18R were similar in blood and LN, but in blood proportions were significantly increased in ACS compared with SA patients. This finding can indicate a more activated state of NK cells in the acute stage of CAD.

Despite several differences in lymphocyte composition between LN and blood, the LN did not reveal any further changes in immune status between SA and ACS patients. The few significant changes observed in blood were not mirrored in LN and the results therefore indicate that the analyses of thoracic LN in patients with advanced CAD do not add any information compared with blood analyses.

Table XII. Distribution of type 1 and 2 lymphocyte subsets in blood and LN.

		Non-STEMI (=13)	SA (=13)	P
CCR4⁺ % of CD4⁺				
Type 2 like	Blood	12 (9.0-19)	14 (11-20)	NS
	LN	0.5 (0.2-0.8)***	0.5 (0.2-0.7)***	NS
IL-18R⁺ % of CD4⁺				
Type 1 like	Blood	6.3 (3.9-7.7)	4.9 (3.2-6.9)	NS
	LN	2.5 (1.8-4.8)*	2.0 (1.5-4.6)	NS
CD3⁺IL-18R⁺ % of CD8⁺				
Type 1 like	Blood	5.3 (2.8-7.5)	3.4 (2.5-4.9)	NS
	LN	5.9 (4.1-25)	6.5 (4.9-15)**	NS
CD3⁺IL-18R⁺ % of NK cells				
Type 1 like	Blood	7.3 (4.4-12)	3.4 (2.7-4.9)	< 0.01
	LN	5.6 (2.1-9.1)	3.7 (2.7-5.5)	NS

Values are given as median (interquartile range). Significant differences between patients with SA and non-STEMI patients were seen only in the proportion of the IL-18R⁺ subpopulation of NK cells in blood. Values * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ refer to differences between LN and blood. NS: not significant $p \geq 0.05$.

NK subpopulations (paper III)

As mentioned (“Major lymphocyte populations paper II-IV”) the proportions of NK cells were decreased in CAD patients compared with healthy controls. Since NK cells are cytokine producers and also exhibit cytotoxic functions, they may have an important role in chronic inflammatory conditions. To further understand the role of NK cells in CAD, we investigated whether the NK deficit was associated with a particular NK subpopulation by using a selection of NK subsets and cytokine profile markers of NK. As shown in Table XIII, no significant differences were seen between patients and controls regarding phenotypic subsets. Also the intracellular cytokine staining of IFN- γ , TNF, IL-10, IL-13 and IL-17A in CD3⁻56^{bright} and CD3⁻56^{dim} cells respectively, showed no significant differences between patients and controls.

Table XIII. Natural killer (NK) cells and subpopulations in CAD patients and controls.

	non-STEMI (n=31)	SA (n=34)	Controls (n=37)	P
NK cells (CD3 ⁻ 16/56 ⁺)	12 (9.8-15) **	12 (9.1-18) *	18 (11-29)	0.01
56 ^{bright} (cytokine producers)	4.4 (3.2-7.3)	3.7 (2.2-6.6)	4.3 (2.6-6.9)	NS
56 ^{dim} (cytotoxic)	96 (93-97)	96 (93-98)	96 (93-97)	NS
56 ^{dim} 57 ⁻ (“early” differentiated)	55 (36-69)	49 (36-66)	46 (31-59)	NS
56 ^{dim} 57 ⁺ (“late” differentiated)	45 (31-64)	51 (34-65)	54 (41-70)	NS
NKAT2 (KIR)	30 (26-37)	27 (21-36)	28 (22-37)	NS
NKB1 (KIR)	6.7 (4.5-11)	6.3 (3.5-15)	6.0 (0.9-12)	NS
NKG2a/CD94 (inhibitory)	33 (27-42)	35 (25-47)	35 (28-45)	NS
56 ⁺ 69 ⁺ (early activated)	2.1 (0.6-5.3)	1.8 (0.8-3.5)	1.3 (0.6-2.3)	NS
56 ⁺ HLA-DR ⁺ (late activated)	4.1 (2.3-8.8)	5.0 (3.0-7.5)	3.0 (1.9-5.5)	NS

NK cells are given as percentages of total lymphocytes. NK cell subsets are given as percentages and refer to percentages of the parent population. Values are given as median (interquartile range). * $p < 0.05$, ** $p < 0.01$ compared with controls. P-value column refers to Kruskal-Wallis test comparing all groups (non-STEMI, SA and controls). NS: not significant $p \geq 0.10$.

Although previous reports showed that expression of activation markers on NK cells are increased in some inflammatory conditions (Evans et al., 2011, Jost et al., 2011) we could not confirm these findings in our study populations; probably the systemic inflammation is too low in CAD or reduced by treatment, to induce these activation markers on NK cells. In contrast, our previous studies showed that NK cells in CAD were more prone to become activated and undergo apoptosis (Jonasson et al., 2005, Li et al., 2008) and also in paper II we found that proportions of NK type 1 cells (IL-18R⁺) were increased in ACS compared to SA patients. Thus, markers have different sensitivities and represent different aspects. Taken together, NK cells in CAD show some aberrations while an extended panel of markers in this study demonstrates that they are not different from controls. Whether the loss of NK cells takes part in the atherosclerosis process or is a consequence, remains to be settled.

Longitudinal follow up of NK cells

The decreased proportions of NK cells in CAD patients (both ACS and SA) increased significantly ($p < 0.01$) during follow up and after 12 months there was no significant difference compared with controls (CAD; median 16%, Controls; median 18 %, $p = 0.191$). Conversely, IL-6 levels were significantly higher in CAD patients at day 1 (CAD; median 3.2 – 4.0 pg/mL, Controls 1.2 pg/mL), but decreased significantly over time ($p < 0.01$ for both patient groups) and showed no difference compared with controls after 3 months (CAD; median 1.6 – 2.3 pg/mL, $p > 0.05$ for both patient groups). After 12 months IL-6 levels were at same levels as at 3 months. However, not all patients showed an increase in NK cells over 12 months. In order to find out if these patients differed in other respects, we divided all CAD patients into tertiles based on percentage change in the NK cell proportion over 12 months and then compared the lower and the upper quartiles, i.e. “non-increase” and “increase”. To establish our thesis of “non-increase” and “increase” in NK cells we had to consider the normal variation over time for this subpopulation. We therefore looked at the variation (presented as CV%) of NK cells over one year in healthy controls by re-calculating data from paper I (Backteman and Ernerudh, 2007) and by using similar data from healthy individuals with samples collected during 2007-2011. As shown in Table XIV, the variation over one year was similar in healthy controls collected during different time periods, as well as in patients with CAD. It is clear that the “increase” group showed a pronounced increase in NK cell proportions, while the “non-increase” group showed no increase or rather a decrease in several individuals. More details regarding variation are presented under “Longitudinal methodological studies” in paper I and III.

Table XIV. Coefficient of variation (CV %) for natural killer (NK) cells over one time in different cohorts; healthy individuals in paper I, healthy individuals with samples collected during 2007-2011, i.e. in parallel with the study in paper III, and in patients with CAD (total and divided into the tertile of the highest increase and the tertile of the lowest (no) increase.

	Healthy controls Paper I (n = 15)	Healthy controls 2007-2011 (n = 7)	CAD patients Paper III Total (n = 43)	CAD patients Paper III “increase group” (n = 14)	CAD patients Paper III “non-increase group” (n = 14)
CV ^a %	22	20	22	83	-11
(range)	(14-30)	(18-22)	(13-30)	(53-97)	(-26-0)

^aMean CV (%) was calculated for NK cells in different longitudinal studies.

After 3 months the IL-6 levels decreased in the “NK increase group” (p<0.01), while no significant decrease in IL-6 was seen in the “NK non-increase group”. The changes in IL-6 led to a lower level in the “NK increase group” (median 1.7 pg/mL) compared with the “NK non-increase group (median 3.0 pg/mL, p 0.003) at 3 months, while there was no difference between the groups at day 1. The NK “increase” *versus* “non-increase” groups did not differ regarding age, sex, history of coronary events or traditional risk factors, except for higher TG levels and waist circumference found in the “non-increase group” (p<0.05 for both parameters), i.e. parameters that are included in the metabolic syndrome. Interestingly, Lynch et al (2009) demonstrated that NK cells are significantly decreased in obese subjects with the metabolic syndrome compared with obese subjects without the metabolic syndrome (Lynch et al., 2009).

Thus, NK cells can have a protective role, while lowered NK cell levels seem to be associated with inflammation and the metabolic syndrome. Of note, NK cells have also been ascribed a protective role in experimental models (Zhang et al., 1997, Flodstrom-Tullberg et al., 2009, Leavenworth et al., 2011). Moreover, in patients with rheumatoid arthritis NK cell numbers are inversely related to disease activity (Lurati et al., 2012). NK cell numbers are also decreased in patients with multiple sclerosis and psoriasis (Perricone et al., 2008, Tobin et al., 2011). Taken together, there is accumulating evidence NK cells can have protective roles in chronic inflammatory conditions. Our studies suggest that this is also the case in CAD.

T cells and subpopulations in relation to CAD and CMV (paper IV)

CD4⁺ T cells lacking expression of CD28 (“CD28^{null}”) have received a lot of attention in CAD, including suggestions of a role in plaque development (Liuzzo et al., 2000, Liuzzo et al., 2007, Dumitriu et al., 2009, Giubilato et al., 2011). However, other factors are involved in differentiating CD4 cells to the CD28^{null} mode, including immunological ageing (immune senescence) and exposure to latent CMV infection. In paper IV we studied CD4⁺ and CD8⁺ T cells regarding loss of the co-stimulatory molecule CD28 as well as regarding expression of the senescent molecule CD57. As shown in Table XV CD4⁺ but not CD8⁺ cells were significantly increased in patient groups compared to healthy subjects. No differences were seen for the CD28^{null} and CD57⁺ subpopulations, neither in CD4⁺ nor in CD8⁺ T cells when comparing across patient groups and controls. However, when all patients and controls were stratified according to CMV seropositivity and seronegativity, the CD28^{null} and

CD28^{null}/CD57⁺ proportions within the CD4⁺ and CD8⁺ populations respectively were increased among the CMV+ individuals (Table XVI). The levels of CMV antibodies were similar across CMV seropositive groups. To further investigate the impact of CMV, we determined intracellular IFN- γ and TNF production in T cells after stimulation with CMV, both in CMV+ and CMV- individuals. The proportions of IFN- γ and TNF were higher in CMV+ individuals, both in CD4⁺ T cells and CD8⁺ T cells as compared with CMV- individuals (Figure 15). There were no differences in IFN- γ and TNF producing cells when comparing CMV+ patients and CMV+ controls. To find out which parameters correlated with the CD28^{null}/CD57⁺ subsets, correlation analyses were performed. The CD4⁺28^{null}, CD4⁺28^{null}57⁺, CD8⁺28^{null} and CD8⁺28^{null}57⁺ subpopulations were all correlated with CMV seropositivity as well as with levels of CMV antibodies. In contrast, only the CD4⁺28^{null} population correlated with the presence of CAD. In addition, CD4⁺28^{null} and CD8⁺28^{null} cells were positively correlated with IL-6 levels in plasma at day 1.

Table XV. CD4⁺ and CD8⁺ cells and CD28/CD57 subpopulations in CAD patients (at day 1) and controls.

	Non-STEMI (n=31)	SA (n=34)	Controls (n=37)	P-value
T helper CD3 ⁺ 4 ⁺	49 (42-56) **	49 (42-54) *	39 (33-49)	0.003
4 ⁺ 28 ^{null}	3.9 (1.2-8.4)	6.1 (1.5-15)	2.9 (0.3-7.3)	NS
4 ⁺ 28 ^{null} 57 ⁺	3.2 (0.8-6.4)	2.0 (0.3-7.5)	1.8 (0.2-6.6)	NS
T cytotox CD3 ⁺ 8 ⁺	25 (19-34)	24 (19-31)	23 (19-29)	NS
8 ⁺ 28 ^{null}	49 (39-66)	49 (23-63)	43 (18-59)	NS
8 ⁺ 28 ^{null} 57 ⁺	32 (25-50)	34 (19-44)	31 (13-51)	NS

CD4⁺ and CD8⁺ T cells are given as percentages of lymphocytes. CD4⁺ and CD8⁺ T cell subsets are given as percentages of CD4⁺ and CD8⁺ T cells, respectively. Values are given as median (interquartile range). * $p < 0.05$, ** $p < 0.01$ compared with controls. The p-value column refers to Kruskal-Wallis test comparing ACS, SA and control groups. NS: not significant, $p \geq 0.10$.

Table XVI. $CD4^+$ and $CD8^+$ subpopulations in CAD patients and controls in relation to CMV status.

	CMV+ (n=79)	CMV- (n=20)	p-value
T helper $CD3^+4^+$	47 (38-52)	46 (42-56)	NS
4^+28^{null}	6.0 (2.9-14)	0.2 (0.0-0.4)	<0.001
$4^+28^{\text{null}}57^+$	3.3 (1.3-9.2)	0.0 (0.0-0.4)	<0.001
T cytotox $CD3^+8^+$	26 (21-33)	18 (14-23)	<0.001
8^+28^{null}	54 (39-66)	19 (15-36)	<0.001
$8^+28^{\text{null}}57^+$	37 (25-53)	11 (6.4-23)	<0.001

$CD4^+$ and $CD8^+$ T cells are given as percentages of lymphocytes. $CD4^+$ and $CD8^+$ T cell subsets are given as percentages of $CD4^+$ and $CD8^+$ T cells, respectively. The values are given as median (interquartile range). The p-value column refers to Mann-Whitney test. NS: not significant, $p \geq 0.05$.

Figure 15. Proportions (%) of IFN- γ and TNF producing $CD4^+$ T cells (A, B) and $CD8^+$ T cells (C, D) in CMV+ individuals (n=27) and CMV- individuals (n=6) in response to CMV peptide stimulation. Proportions are expressed as % of the $CD4$ and $CD8$ populations, respectively. p-values denote differences between CMV+ and CMV- individuals (Mann-Whitney U-test). In addition, when comparing $CD8^+$ and $CD4^+$ T cells, the proportions of IFN- γ^+ and TNF $^+$ cells were significantly higher in the $CD8^+$ T cell population ($p = 0.002$ and 0.007 , respectively, p-values not shown in the graph). Lines indicate median values.

Longitudinal follow up of T cell subpopulations (paper IV)

Follow up samples were taken after 3 and 12 months. Since there were no differences at day 1 between patient groups, results of in total 43 patients (15 ACS patients and 28 SA patients) were pooled together. As shown in Table XVII, the proportions of CD4⁺ T cells significantly decreased after 12 months, although the CD4 fraction was still significantly higher compared with controls ($p < 0.05$). There were no differences during the follow up for total CD8⁺ T cells. Within both CD4⁺ and CD8⁺ T cell populations, CD28^{null} and CD28^{null}57⁺ subpopulations increased significantly at 12 months, though most pronounced in the CD8⁺ population. However, only CD4⁺28^{null} cells were significantly higher compared to controls at 12 months ($p < 0.05$).

Table XVII. Longitudinal follow-up of T cell subpopulations in 43 patients with CAD.

	Day 1	3 months	12 months	P-value
T helper CD3 ⁺ 4 ⁺	50 (42-54) **	48 (42-55)	47 (40-53)	<0.01
4 ⁺ 28 ^{null}	6.0 (1.5-14) **	5.3 (1.8-17)	6.3 (1.8-16)	<0.05
4 ⁺ 28 ^{null} 57 ⁺	2.4 (0.3-8.8) *	3.0 (0.7-14)	3.2 (0.5-14)	<0.05
T cytotox CD3 ⁺ 8 ⁺	24 (19-30) **	24 (20-32)	24 (21-33)	NS
8 ⁺ 28 ^{null}	47 (28-64) ***	57 (30-66) *	61 (29-69)	<0.001
8 ⁺ 28 ^{null} 57 ⁺	31 (17-43) ***	36 (16-46) **	37 (19-55)	<0.01

CD4⁺ and CD8⁺ T cells are given as percentages of lymphocytes. CD4⁺ and CD8⁺ T cell subsets are given as percentages of CD4⁺ and CD8⁺ T cells, respectively. Values are given as median (interquartile range). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with 12 months (Wilcoxon signed rank test). The p-value column refers to Friedmans test for day 1, 3 months and 12 months; NS: not significant, $p > 0.10$.

The current paradigm is that CD28^{null} T cells are associated with unstable plaques (Liuzzo et al., 1999, Liuzzo et al., 2000, Dumitriu et al., 2009, Giubilato et al., 2011), which is reasonable since T cells lacking CD28 expression are large producers of IFN- γ , exhibiting proinflammatory functions. In line with this we found an association between CAD and CD4⁺28^{null} cells and also between IL-6 levels and CD4⁺28^{null} cells at admission. However, the CD4⁺28^{null} cells (as well as CD8⁺28^{null} cells) were much more associated with the presence of latent CMV infection as measured by presence of CMV antibodies, than with the presence of CAD. In support of this, there is a well-known association between loss of CD28/gain of CD57 and CMV seropositivity, as well as with immunological ageing (Liuzzo et al., 1999, Focosi et al., 2010, Strioga et al., 2011, Meijers et al., 2013). Since the CD28^{null}/CD57⁺ subsets increased over time, despite statin treatment, it may be speculated that these changes are reflecting a process of accelerated immunological ageing after coronary event. Concerning correlations to cardiovascular risk, CMV infection itself has not shown consistent results. Blum et al suggest an association between CMV infection and atherosclerosis while other do not support this theory (Blum et al., 2003, Arcari et al., 2005, Xenaki et al., 2009, Alavi et al., 2011). In this study we found no evidence of CMV re-activation in CAD patients. Taken together, CMV was shown to be the major determinant of CD28^{null} and CD57⁺ T cell expansions. Data further suggest that CMV has an impact on the accelerated T cell ageing process that might occur after a cardiac event.

Summary and Conclusions

The specific findings in this thesis are concluded and commented below.

Paper I

The observed methodological and biological variations in repeated measurements over time are both important factors to take into account. In this paper the methodological variation was in general low, showing as expected that the instrument variation was lower than the variation associated with preparation and gating procedures, factors that should be standardized as much as possible. Also as expected, the numbers showed a higher variation than the proportions. Furthermore, there were no major variations over the year for the main lymphocyte populations, indicating that it is not necessary to take into consideration when samples are taken on an annual basis. As expected, the total variation exceeded the methodological variation since the total variation also includes the biological variation. Still, the total variation over time was modest, showing that flow cytometry and immunophenotyping of major lymphocyte populations is a robust technique for longitudinal studies to be used in clinical and research settings.

Paper II

Despite marked differences in lymphocyte composition between LN and blood, the LN did not reveal any further changes in immune status between SA and non-STEMI patients. The few significant changes observed in blood were not mirrored in LN and our results therefore indicate that the analyses of thoracic LN in patients with advanced CAD do not add any information to blood analyses.

Paper III

The proportions of NK cells were low in both non-STEMI and SA patients confirming previous reports on a lack of NK cells in CAD. However, there were no aberrations in NK cell subset distribution or cytokine profile in CAD. During follow-up, failure to reconstitute NK cells was associated with a persistent low-grade systemic inflammation, as measured by plasma IL-6 levels, supporting a protective role of NK cells in CAD.

Paper IV

An increased proportion of CD28^{null} cells within the CD4⁺ population was associated with CAD, in line with previous studies ascribing a role of CD4⁺CD28^{null} cells in CAD. However, we here demonstrated that CMV was the major determinant of the presence of CD28^{null} and CD28^{null}CD57⁺ T cells in patients with CAD, independent of clinical stage. Findings also suggest that CMV might have an impact on the T cell ageing process that occurred in patients after a cardiac event.

Concluding remarks and future perspectives

This thesis adds to the increasing understanding of associations between lymphocyte subpopulations and CAD and the possible role of lymphocyte populations in disease development. The results can be useful to define markers for subgroups of disease or for monitoring of interventions. In the long-term perspective, if the mechanisms of NK cell perturbations can be further clarified, it would be possible to find treatment targeting this defect.

Since atherosclerosis is considered as an inflammatory process the focus in this thesis has been on T cell and NK cell subsets that have been implicated in atherosclerosis and CAD development in previous studies. A strength with the thesis is that longitudinal studies were performed, in contrast to most previous studies on lymphocyte populations in CAD. The longitudinal design also revealed important information that persistently low NK levels were associated with non-resolved inflammation and, unexpectedly, that proportions of CD28^{null}CD57⁺ subsets increased after the cardiac event. Another strength of the thesis is that methodological aspects were carefully considered.

In paper II we found a 10-fold enrichment of Tregs expressing CD69 in LN compared to blood both for SA and ACS patients. Exactly what this expanded population represent is not clarified, although probably the abundant CD69 expression contributes to the sustained presence of Treg cells in the lymph node. It would be of interest to further examine this population, as well as to assess the presence of the population in blood, to see whether it reflects a state of activation or regulatory function. One way to do so could be by phenotyping and sorting of Tregs combined with functional analyses.

The underlying reason to the decreased levels of NK cells in CAD patients is not yet fully clarified. One possibility is that NK cells are affected by stress factors since psychological stress has been associated with increased proinflammatory activity (Sjogren et al., 2006) and it has also been reported that some CAD patients have high levels of psychosocial stress. Therefore, to further clarify the role of NK cells in CAD patients, an ongoing CAD-project is evaluating NK cells and inflammation markers in correlation to perceived stress.

Another factor that can affect the outcome of NK cells may be “defects” in the bone marrow. Proliferation of NK cells occurs in the bone marrow and is crucial for the amounts in the circulation (Grzywacz et al., 2011). It has been suggested that immunosenescence in part is related to a reduction in NK cell proliferation in the bone marrow (Beli et al., 2014). It would therefore be of interest to study whether deviations of NK cell distribution occur already in bone marrow.

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