THE INFLAMMATORY RESPONSE IN EXTRAVASATED LEUKOCYTES IN PATENTS WITH CORONARY ARTERY DISEASE

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ABSTRACT

Coronary artery disease (CAD) is a manifestation of a chronic inflammation in the coronary arteries. The inflammatory process results in accumulation of monocyte derived cells and formation of atherosclerotic plaques in the intima of the vessel wall. Neutrophils are mainly associated with ruptured plaques and the precise role in CAD is not fully known. Extravasation into local inflammatory sites is coordinated by adhesion molecules and chemokines and transforms the leukocytes into activated tissue dwelling cells. The aim of this thesis was to investigate extravasated monocytes and neutrophils in patients with stable CAD. A skin chamber method was applied in order to induce a local inflammation from which extravasated cells were collected.

Paper I and II describes extravasated monocytes. Patients with CAD had a similar number of extravasated monocytes in the chamber exudate compared to healthy subjects. The expression of CD11b following extravasation was lower in patients with CAD compared to healthy controls. This might result in an increased retention of monocytes at a local inflammatory site. Other markers associated with monocyte extravasation, VLA-4 and CX3CR1, were not altered. Extravasated monocytes were further analyzed for functional alterations. Markers associated with antigen presentation, HLA-DR and CD86, and binding of modified cholesterol, CD36 and scavenger receptor A1 (SR-A1) had an increased expression following extravasation compared to in circulation. Furthermore, the binding of acetylated low density lipoprotein (acLDL) increased following extravasation. Monocytes from patients and controls had a similar functional response. However, the chamber fluid from patients with CAD enhanced the expression of CD36 following in vitro stimulation of mononuclear cells.

Paper III and IV describes extravasated neutrophils. Extravasated neutrophils from patients with CAD had a significantly lower expression of CD11b and a lower production of reactive oxygen species (ROS) following stimulation compared to healthy controls. This might indicate a refractory stage following extravasation in patients with CAD. The gene expression in extravasated neutrophils was assessed by a gene array. A general induction in the IL-1 axis was seen following extravasation and was associated with an increased expression of chemokines. Expression of IL-1R on human neutrophils was confirmed with flow cytometry and electron microscopy and stimulation with IL-1 resulted in CCL and CXCL chemokine gene and protein expressions. Compared to healthy controls, extravasated neutrophils from patients with CAD had significantly increased expressions of CCL20 and CXCL2. This finding indicates that neutrophils may have an immuno-modulatory role at local inflammatory sites and that patients with CAD have a chemokine profile that could enhance the pathological processes in atherosclerosis.

The major findings indicate a potential mechanism for monocyte entrapment at local inflammatory sites. In addition, the local inflammatory milieu in patients with CAD might be pro-atherosclerotic. Neutrophils from patients with CAD had an altered responsiveness and could be refractory. Furthermore, neutrophils may alter the local inflammatory milieu by the production of chemokines.
PUBLICATIONS


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## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>ACE</td>
<td>Angiotensin converting enzyme</td>
</tr>
<tr>
<td>acLDL</td>
<td>Acetylated low density lipoprotein</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
</tr>
<tr>
<td>CABG</td>
<td>Coronary artery bypass graft</td>
</tr>
<tr>
<td>CAD</td>
<td>Coronary artery disease</td>
</tr>
<tr>
<td>CGD</td>
<td>Chronic granulomatous disease</td>
</tr>
<tr>
<td>CR</td>
<td>Complement receptor</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>DCFH-DA</td>
<td>Dichlorofluorescin diacetate</td>
</tr>
<tr>
<td>FcR</td>
<td>Fc receptor</td>
</tr>
<tr>
<td>fMLP</td>
<td>N-formylmethionyl leucyl phenylalanine</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte colony stimulating factor</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>HOCL</td>
<td>Hypochlorous acid</td>
</tr>
<tr>
<td>HSA</td>
<td>Human serum albumin</td>
</tr>
<tr>
<td>ICAM</td>
<td>Inter cellular adhesion molecule</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL-1Ra</td>
<td>Interleukin-1 receptor antagonist</td>
</tr>
<tr>
<td>IRAK</td>
<td>Interleukin-1 receptor-associated kinase 1</td>
</tr>
<tr>
<td>LAD</td>
<td>Leukocyte adhesion deficiency</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LFA</td>
<td>Lymphocyte function associated antigen</td>
</tr>
<tr>
<td>MAC</td>
<td>Macrophage antigen</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinases</td>
</tr>
<tr>
<td>MCP</td>
<td>Monocyte chemotactic protein</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescent intensity</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage inflammatory protein</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>NADP</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor kappa light chain enhancer of activated B cells</td>
</tr>
<tr>
<td>NGAL</td>
<td>Neutrophil gelatinase associated lipocalin</td>
</tr>
<tr>
<td>NS</td>
<td>Non-significant</td>
</tr>
<tr>
<td>oxLDL</td>
<td>Oxidised low density lipoprotein</td>
</tr>
<tr>
<td>PAF</td>
<td>Platelet activating factor</td>
</tr>
<tr>
<td>PCI</td>
<td>Percutaneous coronary intervention</td>
</tr>
<tr>
<td>PECAM</td>
<td>Platelet endothelial cell adhesion molecule</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear</td>
</tr>
<tr>
<td>PSGL</td>
<td>P-selectin glycoprotein ligand</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>sIL-1RII</td>
<td>Soluble interleukin-1 receptor type II</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutases</td>
</tr>
<tr>
<td>SRA</td>
<td>Scavenger receptor A</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factors</td>
</tr>
<tr>
<td>UA</td>
<td>Unstable angina</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular cell adhesion molecule</td>
</tr>
<tr>
<td>VLA</td>
<td>Very late antigen</td>
</tr>
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</table>
1 INTRODUCTION

The inflammatory process is crucial in the defense against microorganisms and in the healing of damaged tissue and ceases once the causative mediator is eliminated. However, the inflammatory reaction can become chronic and contribute to various pathological disorders. During a chronic inflammation, the processes that normally regulate defense and healing instead induce tissue destruction and fibrosis. Coronary artery disease (CAD) is characterized by a chronic inflammatory process which leads to formation of atherosclerotic plaques in the blood vessels that provide the heart with oxygen. This thesis has focused on the initial stages of an inflammatory reaction in patients with CAD and healthy subjects.

1.1 GENERAL ASPECTS OF INFLAMMATION

The inflammatory process is defined by five characteristic signs that describe the functional alterations that occur in the tissue: calor (heat), dolor (pain), rubor (redness), tumor (swelling) and function laesa (loss of function). Alterations in the blood vessels in proximity to the injury, such as vascular dilatation and increased permeability, induce an increased blood flow which causes redness and heat. The increased permeability enables plasma and leukocytes to accumulate at the site of injury which causes swelling. Local pain is mediated by inflammatory components that increase the sensitivity of peripheral pain receptors. Pain is a danger signal for the host and contributes to the loss of function. Additional changes include an increased expression of adhesion molecules on activated endothelial cells, which mediates leukocyte extravasation, and clotting of small blood vessels in proximity to the injury, which enclose the inflammatory location.

The establishment of an inflammatory reaction is mediated by activation of tissue resident cells, such as mast cells and macrophages that release mediators which affect the local blood vessels. The first reaction during inflammation is the innate response; it acts within minutes without adaptation. The cellular components of the innate immunity include phagocytic cells, such as neutrophils and monocytes/macrophages. The phagocytic cells circulate in blood and extravasate into injured or infected tissue through a gradient of local inflammatory mediators. At the local inflammatory site they bind to microbes and internalize them in a process called phagocytosis. The internalized microbe is then killed by fusion with granules containing microbicidal proteins and by reactive oxygen species (ROS) that are generated by a process named oxidative burst. The innate response is crucial and defects in this system can cause severe immunodeficiency.

The first line of defense is not always enough to eliminate infectious microorganisms. A second line of defense has therefore evolved which provide a more fine tuned response. The adaptive response is fully activated after four to seven days and includes leukocytes with receptors that specifically recognize the foreign antigen. Hence, a versatile reaction to the specific antigen is induced by the adaptive response. The onset of adaptive immunity is coordinated by the innate responses and depends on innate recognition of the pathogen. The cellular components of adaptive immunity include
antigen presenting cells such as dendritic cells, macrophages and B-lymphocytes and effector cells such as B- and T- lymphocytes. Antigen presenting cells process the foreign antigen and present it to the effector cells that subsequently become activated. B-lymphocytes account for the humoral immune response that generates antibodies and T-lymphocytes account for cell mediated immunity and provide signals that activate B-lymphocytes. In addition, adaptive immunity generates an immunological memory that reinforces the onset and magnitude of the defense at re-infections with the same pathogen.

Leukocytes differentiate from pluripotent stem cells in the bone marrow into two main cell lines. The myeloid lineage gives rise to monocytes, mast cells and granulocytes and the lymphoid lineage gives rise to lymphocytes. Dendritic cells can mature from both types of precursors. Acute inflammatory reactions are associated with neutrophils and monocytes/macrophages. A chronic inflammation on the other hand is mainly driven by monocytes/macrophages and lymphocytes.

1.2 NEUTROPHILS

Neutrophils, together with eosinophils and basophils, belong to a group of leukocytes designated as granulocytes, since they contain intracellular granules. Neutrophils are also called polymononuclear (PMN) cells due to their segmented nucleus that is divided into several lobes.

After approximately five days of proliferation and final maturation, mature neutrophils are released from the bone marrow to the peripheral blood [Bainton 1999]. Neutrophil trafficking from bone marrow is regulated by granulocyte colony-stimulating factor (G-CSF) that has also been endowed to reduce neutrophil apoptosis at local inflammatory sites [Gregory et al 2007, Semerad et al 2002]. Neutrophils comprise approximately 50-70% of the leukocytes in peripheral blood and circulate for about ten hours after which they die. They patrol the blood vessels for inflammatory signals and cease to circulate and migrate into inflamed tissues. The extravasation is regulated by chemokines such as CXCL8 (IL-8). The lifespan of neutrophils during inflammation is enhanced due to expression of survival signals [Altznauer et al 2004]. However, after approximately three days, the neutrophils undergo apoptosis and are cleared from the inflammatory site by macrophage mediated phagocytosis. Clearance may be enhanced by the release of lysophosphatidyicholine from apoptotic cells that augments the extravasation of monocytes [Lauber et al 2003]. By phagocytosis of apoptotic neutrophils, the neutrophil bactericidal proteins are transferred to the macrophages which enhance the macrophage mediated defense against intracellular pathogens [Tan et al 2006]. Clearance of redundant cells by apoptosis and macrophage phagocytosis is a prerequisite for resolution of inflammation. Pus, which is formed during some bacterial infections, is mainly composed of neutrophils and neutrophils’ remnants.

Neutrophils are the first cells to extravasate into inflammatory sites. During the process of extravasation they are transformed to activated tissue dwelling defenders. A key factor in this transformation is the fine tuned sequenced release of granules and secretory vesicles. Once activated, neutrophils phagocytose foreign antigen and exert
their bactericidal actions through production of ROS and release of toxic granule components. Interaction with the target antigen can be mediated by sugar residents that bind to lectins on the bacterial surface [Ofek and Sharon 1988] or via receptors that bind to opsonised particles. Opsonisation can be mediated by antibodies, usually IgG, or components of the complement system, usually C3b, C3bi and C4b, which engage Fc receptors (FcR) and complement receptors (CR), respectively. Phagocytosis of the pathogen is controlled by receptor mediated signaling that results in rearrangement of the cytoskeleton [Strzelecka et al 1997]. Complement receptors that mediate neutrophil phagocytosis include CR1 and CR3 [Greenberg 1999]. CR3 is also known as the adhesion molecule CD11b/CD18 and have therefore dual roles. FcγRIII, also called CD16, is highly expressed on neutrophils and can be used to discriminate neutrophils from other leukocytes.

During differentiation in the bone marrow, neutrophils are armed with effector molecules. These are packed within cytoplasmic stores, sequentially formed during differentiation in the following order: azurophil granules (primary), specific granules (secondary), gelatinase granules (tertiary) and secretory vesicles [Faurschou and Borregaard 2003]. The content of the granules is regulated by the sequential timing of the molecular biosynthesis during the time of granule development. The granules contain bactericidal proteins such as cationic defensins and the bactericidal/permeability increasing protein, which disrupt the negatively charged bacterial cell wall. Granules also contain serin proteases and matrixmetalloproteinases (MMPs) with bactericidal and proteolytic functions. In addition, the granule membranes contain receptors for pattern recognition and phagocytosis, adhesion molecules and components required for the oxidative burst. Simplified, azurophil and specific granules contain bactericidal and proteolytic proteins that are released intracellularly by fusion with phagocytic vesicles [Joiner et al 1989]. However, the specific granules can also be released extracellularly [Sengelov et al 1995]. The release of bactericidal and matrix degrading proteins from gelatinase granules occur mainly extracellularly [Mollinedo et al 1997]. Secretory vesicles are easily translocated to the plasma membrane [Borregaard et al 1987]. They contain plasma proteins and membrane receptors associated with adhesion and pattern recognition, such as CR1, CR3 and CD16, and they

It was long thought that fully differentiated neutrophils were transcriptionally silent. However, a recent publication suggests that activation of biosynthesis occurs following extravasation [Theilgaard-Monch et al 2006]. This transcriptional activation includes induction of cytokines and chemokines [Theilgaard-Monch et al 2004, Coldren et al 2006]. Hence, neutrophils may tune the subsequent inflammatory response. Theilgaard-Monch et al demonstrated that extravasated neutrophils had little transcriptional activity in genes categorized for bactericidal defense, extravasation or changes in cell structure [Theilgaard-Monch et al 2004]. In contrast, a substantial transcriptional activation occurred in genes regulating apoptosis, leukocyte recruitment, proliferation, angiogenesis and modulation of extracellular matrix. The transcriptional activator, nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), is a key factor for gene expression following stimulation with inflammatory agents such as interleukin 1 (IL-1) in human neutrophils [Malcolm et al 2003, McDonald et al 1997, McDonald and Cassatella 1997].
Neutrophils can regulate the subsequent extravasation of other leukocytes by the production and release of chemotactic substances. This has been demonstrated in neutropenic mice that had a reduced extravasation of monocytes that were restored by administration of secretion products from neutrophils [Soehnlein et al 2008]. Neutrophils modulate the local inflammatory milieu by synthesis of chemokines [Scapini et al 2000] and by protease mediated modification of already present chemokines [Padrines et al 1994, Berahovich et al 2005]. In addition, some granule components such as LL-37 are chemotactic for neutrophils and mononuclear cells [De et al 2000].

In recent years it has also been demonstrated that neutrophils influence the adaptive immune response through transport and presentation of antigens in lymphoid tissue [Appelberg 2007]. It is becoming evident that neutrophils, that are the major leukocyte population in peripheral circulation, have far reaching effect beyond the role as a phagocyte and may set the tune for the subsequent events once the inflammatory process has commenced.

1.3 MONOCYTES AND MACROPHAGES

Monocytes comprise 5-10% of the peripheral circulating leukocytes. They develop in the bone marrow, circulate in peripheral blood and migrate into tissue during steady state and inflammation [van Furth 1985]. Extravasated monocytes differentiate into either macrophages or dendritic cells, and this transition is a dynamic process. These cells are therefore commonly termed monocyte derived. Monocyte derived cells are particularly interesting since they mediate innate responses and are a prerequisite for initiation of adaptive immunity. In addition, monocyte derived cells have a longer survival than neutrophils.

Approximately 90% of the circulating monocytes have a high expression of CD14 and lack expression of CD16; these cells are therefore termed CD14+CD16-. In contrast, the CD14+CD16+ monocytes account for approximately 10% of the circulating monocytes [Passlick et al 1989]. The CD14+CD16+ monocytes are proposed to be more differentiated than the CD16 monocytes [Ziegler-Heitbrock et al 1993, Ancuta et al 2000] and it might be that the CD16+ monocytes more readily migrate to inflammatory sites. Recent data indicates that CD16+ and CD16- monocytes may give rise to different macrophage and dendritic cell progeny [Ancuta et al 2009]. However, there is still much to unravel about the subtypes of monocytes. A role for the CD14+CD16+ monocytes in patients with CAD and on hemodialysis has been put forward [Schlitt et al 2004, Nockher and Scherberich 1998]. The CD14+CD16+ monocytes have an increased expression of pro-inflammatory cytokines, increased antigen presentation and reduced phagocytosis and oxidative burst [Ziegler-Heitbrock 2007, Ziegler-Heitbrock 1996].

Macrophages are tissue resident cells, part of the stromal network and associate with the endothelium and epithelium. They are rapidly activated in case of tissue injury or bacterial encounter, and provide activation signals to the endothelium. Macrophages are
constitutively recruited from circulating blood monocytes [Gordon 1999]. Early monocyte differentiation is influenced by adhesive events during extravasation and by the local inflammatory milieu [Wesley et al 1998, Sudhakaran et al 2007, Wang et al 2001, Chomarat et al 2000]. Resident macrophages are phenotypically diverse and differentiate according to their local environment. They can be categorized into two main subtypes, classically activated M1 and alternatively activated M2 [Martinez et al 2006]. However, these subtypes do not reflect the different roles of macrophages in tissue and the M2 group is further divided into subgroups. In a recent paper, Moser et al [Moser and Edwards 2008] divides macrophages into three categories, classically activated, wound healing and regulatory and suggests that the phenotype of macrophages is highly plastic and that all kinds of transitional stages can occur.

Monocytes are transiently modified by the cytokine environment which is influenced by the local influx of leukocytes. The function of the CD14^+CD16^- and the CD14^+CD16^+ monocytes and to what degree these replenish macrophage and dendritic cell populations is not fully understood and needs further studies to be delineated. During differentiation, monocyte derived cells acquire an increased ability for antigen presentation, which is seen as an up-regulation of human leukocyte antigen (HLA) and the co-stimulatory molecules CD80 and CD86 [Santin et al 1999, Laupeze et al 1999].

Compared to neutrophils, monocyte derived cells have a slower onset of extravasation during the initial stages of inflammation. Like neutrophils, they participate in phagocytosis and in oxidative burst [Dale et al 2008]. Macrophage phagocytosis induces clearance of apoptotic cell and microorganisms and provides the macrophage with antigens to be presented. Hence, macrophages are involved in tissue homeostasis as well as in innate and adaptive immunity. Macrophage phagocytosis is mediated by carbohydrates, FcR and CR that bind antigens in a similar manner as in neutrophils. Macrophages also express scavenger receptors that bind to polyanionic ligands and mediate clearance of apoptotic cells and cellular debris. The class B scavenger receptor, CD36, is utilized in clearance of apoptotic cells [Fadok et al 1998]. In addition, macrophages express toll like receptors that are involved in pattern recognition and signal the presence of virus and bacteria.

Following stimulation, macrophages release IL-1β and tumor necrosis factor α (TNF-α), both with profound effects on endothelial cell activation [Furie and Mc Hugh 1989]. Macrophages also release IL-6 and IL-6, IL-1β and TNF-α induce an acute phase response in the liver. An indicator of the acute phase is the increase in systemic C-reactive protein (CRP). Macrophages also produce chemokines, such as IL-8, macrophage inflammatory proteins 1α and 1β (MIP-1α and 1β), monocyte chemotactic protein-1 (MCP-1) and RANTES that induce further leukocyte extravasation.

### 1.4 CONSECUTIVE STEPS IN THE EXTRAVASATION PROCESS

Extravasation occurs primarily at post capillary venules, which are small vessels with a thin layer of smooth muscle cells. Leukocyte extravasation in vivo is divided into several consecutive steps: rolling, activation, firm adhesion and transmigration. In recent years these steps have been refined. Ley et al presented a model that included...
capture, rolling, slow rolling, arrest, adhesion strengthening, crawling and paracellular or transcellular transmigration [Ley et al 2007].

**Figure 1. The consecutive steps of extravasation.**

1.4.1 Endothelial activation

Activation of the endothelium occurs in two steps during an inflammatory response [Pober and Sessa 2007]. The initial activation is mediated by G-protein coupled receptors such as the histamine receptor. The outcome of this activation is opening of endothelial gap junctions, exocytosis of P-selectin and production of platelet activating factor (PAF). The subsequent activation is mediated by TNFα and IL-1 which induce the transcription factors NFκB and activator protein 1 that regulate the production of adhesion molecules and chemokines. In addition, migrating leukocytes activates the endothelium and induces opening of endothelial gaps [Hixenbaugh 1997].

1.4.2 Selectin mediated steps

The initial rolling contact with the endothelium is mediated by selectins which contain lectin domains that bind to carbohydrates. L-selectin (CD62L, LAM-1) is expressed on most circulating leukocytes, whereas E-selectin (CD62E, ELAM-1) and P-selectin (CD62P, LECAM-3) are expressed on endothelial cells during inflammation. Following inflammatory activation, P-selectin is mobilized from intracellular stores [McEver et al 1989] and the production of E-selectin is initiated in endothelial cells [Bevilacqua et al 1987]. The ligand for P-selectin, P-selectin glycoprotein ligand-1
(PSGL-1), mediates leukocyte rolling. PSGL-1 can also bind to L-selectin and thereby mediate secondary capture by which rolling leukocytes capture each other [Paschall and Lawrence 2008]. The interactions during rolling are affected by shear stress that modulates the strength of selectin interactions. This is one explanation for the attachment and detachment that characterize rolling [Marshall et al 2003].

1.4.3 Integrin mediated steps

Integrins are heterodimers consisting of one α and one β chain. The integrin family is continuously growing and there are at least 18 α chains and eight β chains that generate 24 different heterodimers sorted into different subfamilies according to their β subunit [Takada Gen et al 2007]. The extravasation of monocytes and neutrophils is particularly associated with β1 and β2 integrins. The β1 integrins bind to components in the extracellular matrix such as laminin, collagen, fibronectin and vitronectin. The β2 integrins can bind to matrix components, but are more involved in cell-cell contacts. Integrins mediate firm adhesion to endothelial cells. However, integrin mediated rolling during inflammatory conditions has been reported [Gaboury and Kubes 1994, Dunne et al 2002, Berlin et al 1995]. Rolling and firm adhesion by β2 integrins might be synchronized by altering the affinity for the counter receptor on the endothelium [Salas et al 2004].

The β1 integrin α4β1 (very late antigen-4 (VLA-4)) is mainly associated with mononuclear cells. VLA-4 binds to vascular cell adhesion molecule-1 (VCAM-1) on activated endothelial cells [Chuluyan and Issekutz 1993]. VLA-4 is normally not expressed on neutrophils but can be induced by nitric oxide [Conran 2003]. Although VLA-4 may not be directly involved in neutrophil adhesion, a cross talk between β1 and β2 integrins has been suggested [van den Berg et al 2001].

There are three main β2 integrins: αLβ2 (CD11a/CD18, lymphocyte function-associated antigen 1 (LFA-1)), αMβ2 (CD11b/CD18, macrophage-1 antigen (MAC-1), CR3) and αxβ2 (CD11c/CD18, CR4). The β2 counter-receptor on activated endothelial cells is intracellular adhesion molecule 1 (ICAM-1) [Diamond et al 1990, Smith et al 1989]. LFA-1 can also bind to ICAM-2, which is constitutively expressed on resting endothelium [de Fougerolles et al 1991]. Hence, ICAM-2/LFA-1 interaction can mediate leukocyte migration without inflammatory activation while ICAM-1 interactions with β2 integrins are dependent on inflammatory activation. The CD11b/CD18 and CD11c/CD18 integrins may also bind to fibrinogen and complement fragment iC3b and have several overlapping functions.

Integrin mediated adhesion is regulated by altering the amount and affinity of the integrins. Neutrophil and monocyte activation is associated with up-regulation of CD11b/CD18 through mobilization of intracellular granules [Borregaard et al 1994, Miller 1987]. The high affinity conformation of integrins is induced by chemokines. The α-chain of many integrins contains a metal ion binding I-domain that can form an inactive closed conformation or an active and open conformation [Takada et al 2007]. In addition, the β-chain contains an I-like domain with similarities to the I-domain. Chemokines induce an inside out signaling that results in phosphorylation of the β chain and hence dissociation from the α- chain [Takada et al 2007]. This is dependent
on the rearrangement of the cytoskeleton which is mediated by talin that binds the cytoplasmic tail of the \( \beta \) chain [Ratnikov et al. 2005]. The high affinity form is hence associated with separation of the \( \alpha \) and \( \beta \) chains as well as elongation of the integrin conformation.


The importance of integrins in innate immunity is indicated by the leukocyte adhesion deficiency (LAD) syndrome that is associated with defects in the \( \beta_2 \) chain [Kishimoto et al. 1989]. The symptoms of LAD are associated with reduced adherence dependent functions such as chemotaxis, phagocytosis and oxidative burst.

### 1.4.4 Diapedesis

Preceding transmigration, the leukocytes crawl on the endothelium seeking the right location to penetrate. Most migration occurs between endothelial cells through cell junctions and involves adhesion molecules in the immunoglobulin superfamily such as platelet endothelial cell adhesion molecule 1 (PECAM-1) and ICAM, as well as CD99 [Muller et al. 1993, Ley et al. 2007]. A transcellular route engaging ICAM-1 has also been defined [Millan et al. 2006].

### 1.4.5 Extracellular matrix and the basement membrane

The extracellular matrix comprises the connective tissue. The basal membrane, which is a sheath of matrix underneath the endothelium or epithelium, is mainly composed of collagen IV and laminin. Both monocytes and neutrophils have been shown to migrate over the basement membrane preferentially at locations with thinner protein content. The extracellular release of proteases during extravasation could indicate that leukocyte migration is associated with matrix degradation [Sengelov et al. 1995], however, this is not fully known. Neutrophils, in contrast to monocytes, have recently been shown to mediate matrix degradation during in vivo extravasation in a mouse model [Voisin et al. 2009]. Adhesive contact to the extracellular matrix is mediated by \( \beta_1 \) integrins and migration is directed towards gradients of chemokines bound to heparan sulfate proteoglycans [Celie et al. 2009].

### 1.5 CHEMOATTRACTANTS

Chemoattractants induce extravasation and regulate leukocyte functions. Chemoattractants can be products from bacteria, fragments of complement, phospholipid metabolites and chemokines. Bioactive lipids with chemotactic activity include leukotrienes and PAF, these are not further discussed.
1.5.1 fMLP

Formylated peptides released from bacteria and disrupted mitochondria are strong inducers of leukocyte extravasation [Schiffmann et al 1975]. The bacterial tripeptide N-formyl-methionyl-leucyl-phenylalanine (fMLP) is commonly used to study leukocyte chemotaxis and activation. fMLP bind to two receptors with different affinities [Fu et al 2006, Ye et al 1992]. The receptors are located in the plasma membrane and in intracellular granules that are mobilized during inflammation [Sengelov et al 1994]. The receptors are coupled to G-proteins and ligand binding initiates several pathways, among them activation of phosphoinositide 3- kinase, phospholipase C (PLC), protein kinase C (PKC), mitogen activated protein kinases (MAPK), Ca2+ mobilization, small GTPases, NFκB and others [Selvatici et al 2006, Panaro et al 2006, Browning et al 1997]. Activation by fMLP induces many different cellular functions including migration, degranulation, oxidative burst and cytokine production.

1.5.2 C5a

The complement system is activated by microbial encounter. However, a cross talk between complement and coagulation has been reported [Huber-Lang et al 2006]. In addition, phagocytic cells have been shown to generate C5a independently of the traditional mechanisms for complement activation [Huber-Lang et al 2002]. The complement cascade triggers consecutive proteolytic cleavages that ultimately produce biologically active peptides with chemotactic, opsonising or cytolytic functions [Fernandez et al 1978]. C5a is one of the most important products and induces various signaling pathways by binding to G-protein coupled receptors [Monk et al 2007].

1.5.3 Chemokines

Chemokines contain repeated cysteins connected by disulfide bonds and the position of the first two cysteins divide the chemokines into different groups. CC-chemokines have adjacent cysteins while CXC and CX3C chemokines have cysteins separated by one or three amino acids, respectively. Chemokines with a function during inflammation are induced by IL-1, TNF, microbial encounter or phagocytosis [Thorburn et al 2009, Hachicha et al 1998]. In addition, endothelial cells produce chemokines following leukocyte interaction [Lukacs et al 1995]. Chemokines are produced by resident cells such as macrophages and mast cells, endothelial cells, epithelial cells, fibroblasts and by extravasated leukocytes. Chemokines bind to sugar residents on endothelial cells and in the extracellular matrix and are thereby gradually captured at the site of inflammation [Johnson et al 2005]. The importance of a gradient has been demonstrated by intravenous administration of CXCL8 (IL-8) that blocked leukocyte chemotaxis during a dermal inflammation [Hechtman et al 1991].

1.5.4 Regulation of chemokine activity

There are many different chemokines with overlapping functions and they can be classified as inflammatory, homeostatic or with dual-functions [Moser et al 2004]. In addition, many chemokines can bind to several overlapping receptors. Overlapping receptors with different affinity have been reported in for the CXC chemokines [Loetscher et al 1994]. Chemokines can also be modified by gelatinase that has been shown to increase the activity of IL-8 by truncation. The increased activity was more
pronounced for CXCR1 compared to CXCR2 [Van den Steen et al 2000]. CCL chemokines are also modified by truncations. Many chemokines form dimers and this might be an additional way of regulation. Dimerisation is important for suppression of adherence dependent oxidative burst by IL-8 in neutrophils [Williams et al 2005].

### 1.5.5 Chemokine signaling

Chemokines bind to heterotrimeric G-protein coupled receptors. The signaling cascades include activation of PLC, phosphoinositide 3-kinase, c-Src family of tyrosine kinases, Ca$^{2+}$ mobilization, PKC and MAPK, among others [Thelen 2001, Thelen and Stein 2008]. Chemokines account for the inside out signaling that alters the integrin affinity, regulates migration and prime the leukocyte for degranulation and oxidative burst. Although fMLP and chemokines initiate similar signaling pathways, induction of oxidative burst in neutrophils indicate that fMLP and IL-8 may exert their functions through different mechanisms [Fu et al 2004]. In addition, fMLP and IL-8 activate integrins differently during chemotaxis in vitro [Heit et al 2005].

A hierarchy between different attractants exists and end target chemoattractants, such as fMLP and C5a, dominates over endogenous chemoattractants, such as IL-8 and leukotriens [Campbell et al 1997, Heit et al 2002]. The hierarchy between end target and endogenous chemoattractants is partly mediated by their different means of intracellular signaling and the engagement of different adhesion molecules [Heit et al 2002]. The hierarchy ensures that exogenous signals dominate over host derived signals.

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>Category</th>
<th>Receptor</th>
<th>Receptor expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL2 (MCP-1)</td>
<td>Inflammatory</td>
<td>CCR2</td>
<td>Monocytes, Basophils, T-lymphocytes$^+$</td>
</tr>
<tr>
<td>CCL3 (MIP-1α)</td>
<td>Inflammatory</td>
<td>CCR1</td>
<td>Monocytes, Basophils, T-lymphocytes$^+$</td>
</tr>
<tr>
<td>CCL4 (MIP-1β)</td>
<td>Inflammatory</td>
<td>CCR5</td>
<td>Monocytes, T-lymphocytes$^+$, Dendritic cells</td>
</tr>
<tr>
<td>CCL20 (MIP-3α, LARC)</td>
<td>Dual</td>
<td>CCR6</td>
<td>T-lymphocytes$^+$, B-lymphocytes, Dendritic cells</td>
</tr>
<tr>
<td>CXCL2 (MIP-2α, GRO-β)</td>
<td>Inflammatory</td>
<td>CXCR2</td>
<td>Neutrophils, Monocytes</td>
</tr>
<tr>
<td>CXCL8 (IL-8)</td>
<td>Inflammatory</td>
<td>CXCR1</td>
<td>Neutrophils</td>
</tr>
<tr>
<td>CX3CL1 (Fractalkine)</td>
<td>Inflammatory</td>
<td>CX₃CR1</td>
<td>Monocytes/M acrophages, T-lymphocytes$^+$</td>
</tr>
</tbody>
</table>

Table 1. Characteristics of chemokines that have been studied within this thesis.
1.5.6 Fractalkine

Fractalkine (CX3CL1) is the only known member of the CX3C family. Fractalkine is distinguished from other chemokines by the presence of a transmembrane domain by which it binds to endothelial cells. Proteolysis of the membrane bound form generates a soluble form with chemotactic activity. By its different means of expression, fractalkine mediates selectin and integrin independent adhesion as well as chemotaxis [Umehara et al 2004, Imai et al 1997]. Fractalkine is particularly important for the extravasation of the CD14⁺CD16⁺ monocytes [Ancuta et al 2003].

1.6 LEUKOCYTE PRIMING, ACTIVATION AND DESENSITIZATION

Leukocyte responses can be amplified by priming and down-regulated by desensitization. This way of regulating the inflammatory response ensures a rapid beginning and a controlled termination.

1.6.1 Priming and activation

Chemoattractants induce firm adhesion and prime leukocytes for further cytotoxic responses such as oxidative burst. IL-8 priming of fMLP induced oxidative burst includes a sequential gathering of the reduced form of nicotinamide adenine dinucleotide phosphate (NADP), the NADPH oxidase, in neutrophils [Guichard et al 2005]. Priming by C5a mediates partially different pathways for activation of oxidative burst by phorbol 12-myristate 13-acetate (PMA) or E-coli [Wrann et al 2007]. In addition, inflammatory cytokines and β2 integrin cross linking mediates priming of fMLP induced oxidative burst and expression of adhesion molecules [Condliffe et al 1996, Wittmann et al 2004, Elbim et al 1994, Liles et al 1995]. Priming is also associated with inflammatory conditions. Neutrophil priming by immunoglobulin aggregates in synovial fluid has been detected in patients with rheumatoid arthritis [Robinson et al 1992]. The mechanism of priming is not fully understood and involves several levels of regulation that may differ between different agonists. Potential mechanisms include G-protein mediated events and their downstream signaling pathways as well as tyrosine phosphorylations. Different means of priming has been reviewed by Condliffe et al [1998]. Furthermore, reversible priming has been suggested for PAF which favors the idea of a balance between priming and de-priming in the regulation of neutrophil activity [Kitchen 1996].

1.6.2 Desensitization

G-protein coupled receptors are phosphorylated and internalized following ligand binding and this response mediates a rapid homologous desensitization [UHING and Snyderman 1999]. Regained responsiveness is regulated by a balance between receptor degradation, de-phosphorylation and hence re-expression as well as synthesis of new receptors. Homologous desensitization has been reported for the receptors of fMLP, C5a, IL-8, leukotrienes, PAF and MCP-1 [Tomhave et al 1994, Franci et al 1996]. Heterologous desensitization that occurs between different G-protein coupled receptors is mediated at the level of second messengers, such as PLC and does not involve ligand binding [UHING and Snyderman 1999]. Heterologous desensitization is dependent on the type of G-protein the respective receptor utilizes. Cross phosphorylation has been
detected between the fMLP, C5a and IL-8 receptors. Desensitization of the C5a and IL-8 receptors is likely to involve PKC [Richardson et al 1995, Tomhave et al 1994]. The fMLP receptor on the other hand has no domains for PKC phosphorylation and desensitization by IL-8 or C5a is therefore likely to involve other mechanisms related to PLC [Richardson et al 1995].

1.7 THE OXIDATIVE BURST, PRODUCTION OF ROS

1.7.1 The NADPH oxidase

Phagocytic cells synthesize toxic oxygen metabolites as part of their effector functions. The first reaction is mediated by the NADPH oxidase that catalyses the reduction of O2 into the superoxide anion (O2-). The NADPH oxidase is composed of membrane and cytosolic components that translocate and assemble in the plasma or granule membranes following activation. Phosphorylation is essential for subunit translocation and partial assembly is seen following priming [Sheppard et al 2005]. However, complete assembly and production of O2- requires full activation. The membrane bound part of the NADPH complex, cytochrome b558, is located to 80-90% in specific granules. These are translocated to the plasma and phagosomal membranes following phagocytosis or stimulation [Borregaard et al 1983, Jesaitis et al 1990]. Chemoattractants mainly induce NADPH oxidase activity in the plasma membrane while phagocytosis induces activity in intracellular granules [Karlsson and Dahlgren 2002]. Production of O2- has also been demonstrated following integrin engagement [Berton et al 1992, Yan and Novak 1999].

The importance of the NADPH oxidase system is indicated in patients with chronic granulomatous disease (CGD). These patients have a defect in superoxide production and suffer from recurrent bacterial and fungal infections [Holmes et al 1967, Quie et al 1967].

1.7.2 Formation of hydrogen peroxide

The superoxide anion is dismutated into hydrogen peroxide (H2O2) and this reaction can be either spontaneous or catalyzed by the superoxide dismutase (SOD) [Klebanoff 1999]. The intracellular production of H2O2 can be measured by flow cytometry using dichlorofluorescein diacetate (DCFH-DA) labeled cells [Bass et al 1983]. The DCFH-DA molecule diffuses over the cell membrane and accumulates intracellularly following desacetylation. The desacetylation product, DCFH, is then oxidized in the presence of H2O2 and the oxidized product is fluorescent. The DCFH-DA system enables a quantitative assessment of the intracellular H2O2 production. PMA, which is a direct activator of PKC and commonly used to study the production of oxygen radicals, induces both intra- and extracellular production of H2O2 in approximately similar amounts [Lundqvist et al 1996].

There are several scavenger systems that degrade H2O2. These include the catalase and the glutathione peroxidase systems, and they protect the host from the toxic effects of H2O2 [Voetman and Roos 1980, Roos et al 1979]. Extracellular release of ROS accounts for tissue destruction and fibrosis associated with chronic inflammation.
1.7.3 Reactions between H₂O₂ and myeloperoxidase

Myeloperoxidase (MPO) is stored in the azurophil granules [Bainton et al. 1971] and catalyses further modification of H₂O₂ into more potent radicals. The main reaction occurs between MPO, H₂O₂ and chlorine and generates hypochlorous acid (HOCL). The MPO/H₂O₂ system can also mediate production of reactive nitrogen intermediates, tyrosyl radicals and halogenation by chloride as well as other halides [Klebanoff 2005]. The products of this system are toxic to a broad spectrum of infectious agent and in addition, they inactivate bacterial toxins [Klebanoff 1999].

Figure 2. Production of reactive oxygen species.

1.8 THE SKIN CHAMBER METHOD

Most studies of human leukocytes utilize cells from peripheral blood or cells collected from local inflammatory sites such as bronchoalveolar lavage (BAL) or synovial fluids. In order to study in vivo activated leukocytes from patients with atherosclerosis, we have used a skin chamber method. This method enables the study of leukocytes that have extravasated to an inflammatory site in vivo [Follin and Dahlgren 2007]. Skin blisters are induced by suction and gentle heating and this separates epidermis from the underlying basal membrane and dermis [Kiiistala and Mustakallio 1964, 1967]. The blister roofs that contain epidermis are removed and plastic chambers are mounted over the exposed wounds. The dermal papillae capillaries are then exposed to an inflammatory stimulation. The inflammatory reaction can be stimulated by autologous serum [Kuhns et al. 1992], zymosan activated autologous serum [Forsgren and Scheja 1985], isotonic salt buffer [Perillie and Finch 1964] or allergens [Nopp et al. 2000]. In the present thesis, we have used autologous serum to induce an intense inflammatory reaction and PBS to induce an intermediate inflammatory reaction.

Leukocyte extravasation into the skin chamber occurs at dermal capillaries and venules and includes interaction with endothelial cells and the extracellular matrix in the basal
membrane. The detached leukocytes are dominated by neutrophils which constitute over 90% of the extravasated cells after 12-24 hours [Kuhns et al. 1992, Koivuranta-Vaara 1985, Forsgren and Scheja 1985]. The extravasation of monocytes is slower and monocytes constitute 15% of the exudated cells after 10 hours.

1.8.1 The inflammatory milieu

Studies with heat inactivated and zymosan activated autologous serum indicate that mediators produced by complement activation are crucial in the inflammatory milieu [Kuhns et al. 1992, Forsgren and Scheja 1985, Scheja and Forsgren 1985]. A major component during the early inflammatory reaction is C5a which is accompanied by IL-8 after a few hours [Follin et al. 1991]. Additional inflammatory mediators in the skin chamber include interferon-γ, leukotriene-B4, IL-6, IL-1β, TNFα and GM-CSF [Kuhns et al. 1992]. The inflammatory milieu in the skin chamber is orchestrated by extravasated leukocytes and dermal mast cells and could be further influenced by fibroblasts and tissue macrophages in the deeper layers of the skin.

1.8.2 Studies of leukocyte function

Extravasated cells can be both primed and desensitized. Desensitization of exudated cells has been noted for C5a and IL-8, the major inflammatory mediators in the chamber exudate [Follin et al. 1991]. Priming has been noted for fMLP and relates to an increased expression of fMLP receptors due to granule mobilization. An increased expression of fMLP receptors and CD11b/CD18, as well as augmentation of fMLP induced oxidative burst, has been reported for exudated neutrophils [Zimmerli et al. 1986]. The absence of priming towards PMA, a stimuli that bypasses receptor activation, indicated a receptor specific response. During extravasation secretory vesicles and granules are mobilized [Wright and Gallin 1979]. The order of mobilization follows in the reverse order of the formation. Extravasation to the skin chamber mobilizes 100% of the secretory vesicles, 40% of the gelatinase granules, 20% of the specific granules and 10% of the azurophilic granules [Sengelov et al. 1995]. Additional alterations in extravasated leukocytes include increased expression of VLA-2 and CD11b/CD18 [Werr et al. 2000, Sengelov et al. 1995] and shedding of L-selectin [Kuhns et al. 1995]. Extravasated neutrophils are also transcriptionally activated [Thielgaard-Monch et al. 2004].

The past decade, the skin chamber model has been applied to evaluate basic leukocyte function following extravasation in healthy individuals [Thielgaard-Monch et al. 2004], patients with allergy [Nopp et al. 2000] and patients with renal disease [Dadfar et al. 2004]. For the first time, we have applied the skin blister model to evaluate functionally activated leukocytes in patients with CAD.

1.9 CORONARY ARTERY DISEASE

The coronary arteries supply the myocardium with nutrients and oxygen. Development of atherosclerotic plaques in the wall of these vessels gives raise to CAD and clinical manifestations such as angina pectoris and myocardial infarction (MI). CAD is the most common cause of acute hospitalization in Sweden and MI is the most frequent
individual cause of death. The incidence of CAD is highly related to age and women have a later onset (8-10 years) than men. Individual risk factors for CAD include smoking, hypertension, obesity, hypercholesterolemia, diabetes mellitus and inherited factors. Arteries with occlusions or stenoses can be treated by percutaneous coronary intervention (PCI) where the vessel lumen is widened by a balloon. This procedure is often associated with the insertion of a metal network called stent to prevent the risk of restenosis. Affected arteries may also be surgically bypassed by a coronary artery bypass graft (CABG). Pharmacological treatments include lipid lowering therapy with statins, antithrombotic therapy (aspirin and clopidogrel), β-receptor blockers and the use of angiotensin-converting enzyme (ACE) inhibitors.

Atherosclerotic lesions within the intima of the vessel wall build up during decades. Small inclusions of fat, called fatty streaks, can be detected in children and with increasing age these inclusions may cause pathological atherosclerotic plaques. Atherosclerosis is associated with an inflammatory reaction in the plaque, but the initial trigger is not fully known. A combination of multiple factors that induce local stress to the coronary arteries such as an altered blood flow, smoking and infections might contribute. Modification of the cholesterol molecule low density lipoprotein (LDL) into oxidized LDL (oxLDL) is crucial for plaque development and contributes to inflammation. Macrophages within the plaque bind to oxLDL and are transformed into lipid loaded foam cells. The coronary arteries have a dense layer of smooth muscle and in advanced stages of atherosclerosis these become activated. The advanced plaque is composed of a lipid core surrounded by a fibrous cap of smooth muscle cells and extracellular matrix. The fibrous cap is protective and breakdown of the cap causes tissue factor in the lesion to leak into the blood, causing the formation of a thrombus. The blockage of coronary arteries by a thrombus initiates myocardial infarction. The atherosclerotic plaque is composed of monocyte derived foam cells, smooth muscle cells and some T-lymphocytes. Occasionally, other cell types such as neutrophils are found.

1.9.1 Monocytes in CAD

Monocyte derived cells are the dominant cell type in the lipid core [Jonasson et al 1986, Bonanno et al 2000]. The monocytes are mainly recruited from the circulation [Lessner et al 2002] by expression of chemokines such as CCL2 in the atherosclerotic plaques [Nelken et al 1991]. The number of circulating CD14⁺CD16⁺ monocytes is slightly increased in CAD compared to in healthy controls [Schlitt et al 2004] and these cells predict the incidence of cardiovascular events in patients on dialysis [Heine et al 2008]. The CD14⁺CD16⁺ monocytes produce substantial amounts of TNFα [Belge et al 2002] and the number of circulating CD14⁺CD16⁺ monocytes associate with the concentration of TNFα in serum [Schlitt et al 2004]. Fractalkine is expressed on human atherosclerotic endothelium which indicates a route of extravasation for the CD14⁺CD16⁺ monocytes [Volger et al 2007, Yano et al 2007]. Knock out studies in mice indicate that fractalkine is important in CAD due to the extravasation of monocyte derived cells which contribute to plaque growth [Lesnik et al 2003]. The expression of fractalkine on smooth muscle cells is up-regulated by oxLDL [Barlic et al 2007] and CX3CR1 signaling may promote the survival of monocytes in the plaque [Landsman et al 2009].
1.9.1.1 Scavenger receptors and modified cholesterol

Following extravasation, monocytes differentiate into macrophages which are transformed into lipid loaded foam cells. A prerequisite in this process is the binding of modified cholesterol to scavenger receptors. The type B scavenger receptor, CD36, is up-regulated during macrophage differentiation [Huh et al 1996] and can be detected on foam cells in human atherosclerotic plaques [Nakata et al 1999]. Many scavenger receptors have the capacity to bind to modified lipids, but CD36 is the main receptor for oxLDL [Endemann et al 1993]. Ligands for CD36 can be generated by the MPO/H2O2/nitrite system [Podrez et al 2000]. CD36 has overlapping affinities with other scavenger receptors, especially scavenger receptor class A1 (SR-A1). Both bind to oxLDL and acetylated LDL (acLDL), but CD36 has highest affinity to oxLDL, while SRA has high affinity to acLDL [Kunjathoor et al 2002]. Following in vitro incubation with modified LDL, smooth muscle cells and endothelial cells increase their expression of CCL2 [Cushing et al 1990] and macrophages increase their expression of CD36 [Han et al 1997]. This implies that LDL mediates several pro-inflammatory mechanisms that are associated with atherosclerosis. In addition, oxLDL induced signaling from CD36 inhibits macrophage migration and could mediate macrophage trapping [Park et al 2009]. The CD14^+CD16^+ monocytes have a lower gene expression of SR-A1 and a similar expression of CD36 as the CD14^+CD16^- monocytes [Draude et al 1999]. However, the binding of modified LDL to CD14^+CD16^+ monocytes has not been fully elucidated [Draude et al 1999, Mosig et al 2009].

1.9.1.2 Antigen presentation

Extravasated monocytes may differentiate into antigen presenting cells, indicated by an increased expression of HLA and co-stimulatory molecules. The co-stimulatory molecules CD80 and CD86 have been detected on macrophages in human atherosclerotic lesions [deBoer et al 1997]. In vitro differentiated dendritic cells from patients with CAD have an increased expression of co-stimulatory molecules compared to cells differentiated from healthy subjects [Dopheide et al 2007]. Knock out studies in mice indicate that CD86 and CD80 are important for activation of T-lymphocytes and development of atherosclerotic lesions [Buono et al 2004]. The presence of activated T-lymphocytes in human plaques further indicates a possible role of smooth muscle cells and macrophages in local activation [Hansson et al 1989]. Furthermore, oxLDL induces the expression of HLA-DR and CD86 on human monocytes and promotes in vitro proliferation of T-lymphocytes [Fortun et al 2001].

1.9.1.3 Monocyte emigration in CAD

During resolution of inflammation, monocytes emigrate from the inflammatory site to peripheral lymph nodes and this process is impaired during atherosclerosis [Randolph 2008]. Emigration of monocyte derived cells is accompanied with lesion regression [Llodra et al 2004] and might involve interactions between ICAM-1 and CD11/CD18 [Randolph and Furie 1996].
1.9.2 Neutrophils in CAD

The role of neutrophils in CAD has not been fully considered, however accumulating data indicates that neutrophils may contribute to the inflammatory reaction [Baetta and Corsini 2009]. A high number of circulating neutrophils is associated with an increased risk of CAD [Horne et al 2005, Haumer et al 2005] and the number of neutrophils correlates to the concentration of pro-inflammatory molecules in circulation [Nijm et al 2005]. Neutrophils are detected at low numbers in fatty streaks in primates [Trillo 1982] and associate with acute coronary events in humans [Naruko et al 2002]. In a mouse model of atherosclerosis, neutrophils were the predominant leukocyte that interacted with atherosclerotic endothelium [Eriksson et al 2001]. Although increasing evidence suggests that neutrophils may infiltrate the atherosclerotic plaque, their short life span in tissue may limit their accumulation. A balance between CXCR2 and CXCR4 mediated signals has been suggested to regulate neutrophil extravasation to atherosclerotic lesions [Sainz and Sata 2008] since antagonists to CXCR4 promoted lesion formation in mice [Zernecke et al 2008]. Furthermore, antibody mediated depletion of neutrophils reduced the plaque area in mice [Zernecke et al 2008]. Together, these results imply that neutrophils contribute to the formation and progression of atherosclerotic plaques. Potential mechanisms for neutrophils in atherosclerosis may be by the release of cytokines, chemokines, MMPs and ROS.

1.9.2.1 Influence on leukocyte extravasation

Once activated, neutrophils secrete both CXCL and CCL chemokines and hence, induce the migration of many different leukocytes. In addition, granule proteins such as LL37 and heparin binding protein are chemotactic for monocytes [Soehnlein et al
Neutrophil interaction with endothelial cells can also induce opening of endothelial gap junctions, thereby promoting leukocyte extravasation. Hence, neutrophils can modulate the inflammatory milieu and the local recruitment of leukocytes to the plaque.

1.9.2.2 Generation of ROS in CAD

ROS, produced by endothelial cells and extravasated leukocytes, have profound effects on the initiation and progression of atherosclerosis. Neutrophils and monocytes that express MPO have been detected in the fibrous cap of unstable plaques [Tavora et al 2009]. Furthermore, the release of MPO during acute manifestations has been detected [Biasucci et al 1996] and the concentration of MPO in plasma correlates to the severity of CAD [Ndrepepa et al 2008]. ROS oxidize LDL and activate endothelial cells and smooth muscle cells. An increased production of ROS following PMA stimulation has been reported in patients with acute coronary disease [Takeshita et al 1997]. This is in contrast to patients with stable CAD who have a lower production of ROS following PMA stimulation [Sarndahl et al 2007].

1.9.2.3 Release of matrix degrading proteases

The release of proteases from activated neutrophils constitutes one mechanism for breakdown and rupture of the fibrous cap. This is indicated by the association of neutrophils with ruptured plaques. The activity of MMP-9 is regulated by the formation of a complex with neutrophil gelatinase-associated lipocalin (NGAL) [Yan et al 2001]. Both NGAL and MMP-9 are expressed in acute CAD and associate with plaque rupture [Hemdahl et al 2005, Fukuda et al 2006].

![Figure 4. The role of neutrophils in CAD with attention to markers studied in this thesis.](image-url)
1.9.3 Integrin profile in CAD

The expression of CD11b on circulating neutrophils and monocytes is increased during acute phases of CAD, which indicates cell activation [Meisel et al 1998, Lindmark et al 2001]. In addition, patients with acute MI have a higher expression of CD11a, VLA-4 and ICAM-1 on the circulating monocytes [Meisel et al 1998]. The expression and the affinity of CD11b are not altered during stable disease [Lindmark et al 2001, Sarndahl et al 2007]. Supporting data for a link between integrin expression and atherosclerosis is indicated by bone marrow transfer in CD18 knock out atherosclerotic mice. This experiment indicated that CD18 could be protective during fatty streak formation and pro-atherogenic in established mature lesions [Merched et al 2009].
2 AIMS

The objective was to study leukocyte in vivo extravasation in patients with CAD and healthy controls.

The specific aims were:
- to study if monocyte extravasation was altered in patients with CAD and hence could explain accumulation of monocyte derived cells in the plaque (I).
- to study if extravasated monocytes were functionally different in patients with CAD compared to healthy controls (II).
- to examine the hypothesis of neutrophil priming in patients with CAD by assessing the responsiveness and activation of peripheral and extravasated neutrophils (III).
- to study if extravasated neutrophils from patients with CAD could contribute to an enhanced pro-atherosclerotic inflammatory milieu (IV).
3 METHODS

This is a general overview of the methods used in the present thesis. For detailed descriptions please refer to each individual article.

3.1 PATIENT CHARACTERISTICS

Patients with stable CAD were recruited from the Karolinska University Hospital. All patients had a history of previous MI or unstable angina (UA), angiographically confirmed atherosclerosis in at least two vessels and normal levels of creatinine in the serum.

<table>
<thead>
<tr>
<th></th>
<th>Females / Males</th>
<th>Age (years)</th>
<th>MI / UA</th>
<th>Delay (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>12 / 7</td>
<td>61 (53-67)</td>
<td>17 / 2</td>
<td>8 (6-12)</td>
</tr>
<tr>
<td>II</td>
<td>7 / 11</td>
<td>62 (52-66)</td>
<td>17/1</td>
<td>11 (8-17)</td>
</tr>
<tr>
<td>III</td>
<td>9 / 4</td>
<td>59 (34-78)</td>
<td>12/1</td>
<td>12 (6-16)</td>
</tr>
<tr>
<td>IV</td>
<td>2 / 8</td>
<td>62 (58-64)</td>
<td>10</td>
<td>9 (8-10)</td>
</tr>
</tbody>
</table>

Table 2. Characteristics of patients included in paper I-IV.

The healthy controls were matched for gender and age ±3 or ±5 years. Patients and controls with known active inflammatory diseases (other than atherosclerosis), infections, diabetes mellitus or rheumatic diseases, as well as those receiving medical therapy with antibiotics, corticosteroids, immunosuppressive agents or Warfarin were excluded. All subjects gave informed consent and the studies were approved by the local ethical committee at the Karolinska University Hospital.

3.2 IN VIVO EXTRAVASATION, THE SKIN BLISTER METHOD

Skin blisters were induced on the forearm by a vacuum of 300 mmHg and heating at 39°C for 2-3 hours. The following morning (after approximately 14 hours), the blister exudates were aspirated and the blister areas were washed with PBS that was added to the collected samples (un-stimulated blister). The blister roofs were removed and sterilized open-bottom plastic skin chambers were mounted over the exposed blister floor. The chambers were filled with PBS (intermediately stimulated blister) or autologous serum (intensely stimulated blister), both containing heparin. After 9-10 hours, the chamber exudates were collected and the skin chambers were washed with an equal volume of PBS that was added to the collected sample. The chamber exudates were centrifuged and the supernatants were frozen and analyzed later. The cell pellets were dissolved in cell culturing medium and directly analyzed.
Figure 5. The skin blister method. Skin blisters are induced by vacuum and gentle heating (A). The un-stimulated blister exudate is collected the following morning after approximately 14 hours (B). After removal of the epidermal roof, skin chambers are mounted over the wound and filled with either PBS or autologous serum, representing the intermediately and intensely stimulated blisters (C).

3.3 ADDITIONAL METHODS

Binding of acLDL (II) Performed by incubation with acLDL conjugated to Alexa Fluor 488 and measured by flow cytometry.

Electron microscopy (IV) Enables visualization of cell morphology and antibody based staining of specific markers. Performed at the electron microscopy unit, Karolinska Institutet.

Ficoll Paque technique (II, IV) Separation of neutrophils and mononuclear cells by density centrifugation.

Flow cytometry
- Analysis of surface markers (I-IV) Epics Elite (Beckman Coulter). Performed by labeling the leukocytes with fluorochrome conjugated antibodies.
- Evaluation of cell count (I-IV) Determines total cell count and distribution of individual cell types.

Immunoassay (I, III, IV) Determines the concentration of pro-inflammatory molecules in serum and chamber exudates by commercially available assays.

In vitro activation (I-IV) Studied following stimulation with fMLP or PMA.
| **In vitro incubations** | Performed on leukocytes from peripheral blood to evaluate the impact of the inflammatory milieu.  
- With serum and chamber exudate (I, II)  
- With cytokines (IV)  
  Neutrophil activation by IL-1 was analyzed by incubations with recombinant human IL-1 after pre-treatment with IL-1R antagonist (IL-1Ra). |
| **In vitro transmigration (I)** | Assessed by the transwell method using inserts coated with extracellular matrix. |
| **Microarray (IV)** | Provides a thorough evaluation of gene expression. Performed at the microarray facility unit, Karolinska Biomics Center. |
| **Oxidative burst (III)** | Assessment of intracellular H₂O₂ production by the 2',7'-dichlorofluorescein diacetate (DCFH-DA) system and flow cytometry. |
| **Purification of RNA (IV)** | Extraction of RNA from purified leukocyte populations by the Qiagen RNeasy kit. |
| **RT-PCR (IV)** | Enables expressional analysis of individual genes. The Taq-Man system was used and genes were detected by means of the FAM-dye labeling system with commercially available primers. The results were normalized against a housekeeping gene and the relative expressions were calculated using the equation 2⁻ΔΔCt. |
| **Statistical analyses (I-IV)** | Comparisons between groups were made by use of Mann-Whitney U test. Correlations were made by Spearman’s correlation analysis. A P<0.05 was considered significant. |
| **Validation of microarray (IV)** | Comparative analysis was carried out using GeneSpring™ (7.3). Data was normalized per chip and per gene. The expression profiles from three independent experiments were compared using ANOVA parametric test, assume variances equal. |
4 RESULTS AND DISCUSSION

4.1 GENERAL CHARACTERIZATION OF THE SKIN BLISTERS (I-IV)

The numbers of extravasated leukocytes, collected with the skin chamber method, are presented in figure 6 which was published in paper I. Similar numbers were detected in paper II-IV.

Figure 6. Numbers of transmigrated leukocytes (x10^3) in the un-stimulated, intermediately stimulated (PBS) and intensely stimulated (serum) blisters. No significant differences between patients and controls were detected. The numbers of transmigrated cells increased significantly with the degree of inflammation.

*** P<0.005 between different blister compartments.

The median percentages of granulocytes, monocytes and lymphocytes in peripheral blood and in the exudates from healthy controls are presented in table 3. Data is adapted from paper I and represents a common distribution. No significant differences in the distribution of extravasated cells were detected between patients with CAD and healthy controls.

<table>
<thead>
<tr>
<th></th>
<th>Granulocytes %</th>
<th>Monocytes %</th>
<th>Lymphocytes %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>60 (52-66)</td>
<td>11 (8.2-12)</td>
<td>30 (26-36)</td>
</tr>
<tr>
<td>Un-stimulated</td>
<td>59 (40-74)</td>
<td>34 (19-46)</td>
<td>7.7 (5.4-12)</td>
</tr>
<tr>
<td>blister</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intermediately</td>
<td>81 (70-87)</td>
<td>13 (9.9-18)</td>
<td>3.5 (2.3-6.9)</td>
</tr>
<tr>
<td>stimulated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>blister</td>
<td>81 (77-86)</td>
<td>14 (10-20)</td>
<td>2.3 (1.3-6.6)</td>
</tr>
<tr>
<td>Intensely</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>stimulated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>blister</td>
<td>81 (77-86)</td>
<td>14 (10-20)</td>
<td>2.3 (1.3-6.6)</td>
</tr>
</tbody>
</table>

Table 3. The median percentage of granulocytes, monocytes and lymphocytes in peripheral blood and the skin blisters. Data represents a common distribution in healthy subjects.
The numbers and distributions of leukocytes in the skin chamber exudates are in the same range as in other publications [Forsgren and Scheja 1985, Hellum and Solberg 1977]. The number of extravasated leukocytes increased with the degree of inflammation and was highest in the intensely stimulated blister. The un-stimulated blister had a higher percentage of monocytes compared to the stimulated chamber exudates which is in agreement with a slower accumulation of monocytes. The un-stimulated blister is incubated overnight for approximately 14 hours while the skin chambers are incubated for 9-10 hours. All exudates were dominated by neutrophils and this is in agreement with previous publications. Figure 7 (from a manuscript not included in the thesis) views the morphology of neutrophils from blood and chamber exudates assessed by electron microscopy. The pictures indicate that extravasated neutrophils have an intact morphology with minor alterations in granule distribution.

![Figure 7](image)

Figure 7. Electron microscopy of peripheral neutrophils (A, C) and extravasated neutrophils (B, D) from a healthy individual.

The concentrations of inflammatory markers in the exudates and in serum are presented in table 4, which is based on immunoassays from paper I, III and IV. Higher concentrations of inflammatory markers were detected in the chamber exudates compared to in serum and in addition increased with the intensity of inflammation. This confirms two separate inflammatory milieus. The numbers of extravasated leukocytes correlated to the ratios of CCL2 and CCL3 concentrations between blister exudates and serum (paper I) which indicates the importance of a gradient.
Table 4. The concentrations of inflammatory markers in exudates and serum. * P<0.05, ** P<0.01, *** P<0.005 between patients with CAD and healthy subjects.

<table>
<thead>
<tr>
<th></th>
<th>Serum</th>
<th>Intermediate blister</th>
<th>Intense blister</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CAD</td>
<td>Healthy</td>
<td>CAD</td>
</tr>
<tr>
<td>IL-6 (I)</td>
<td>(pg/ml)</td>
<td>1.5 (1.1-4.8)</td>
<td>1.3 (0.9-20)</td>
</tr>
<tr>
<td>IL-6 (III)</td>
<td>(pg/mL)</td>
<td>1.5 (1.1-5.2)</td>
<td>1.8 (1.2-3.5)</td>
</tr>
<tr>
<td>IL-8 (III)</td>
<td>(pg/mL)</td>
<td>6.8 (3.7-12)**</td>
<td>1.3 (1.3-5.4)</td>
</tr>
<tr>
<td>IL-10 (I)</td>
<td>(pg/ml)</td>
<td>0.7 (0.3-1.2)</td>
<td>0.5 (0.3-0.9)</td>
</tr>
<tr>
<td>TNF-α (I)</td>
<td>(pg/ml)</td>
<td>1.6 (1.3-3.6)</td>
<td>1.4 (0.7-1.9)</td>
</tr>
<tr>
<td>IL-1α (IV)</td>
<td>(pg/ml)</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>IL-1β (IV)</td>
<td>(pg/ml)</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>IL-1Ra (IV)</td>
<td>(ng/ml)</td>
<td>0.3 (0.2-0.3)</td>
<td>0.2 (0.1-0.4)</td>
</tr>
<tr>
<td>IL-1R2 (IV)</td>
<td>(ng/ml)</td>
<td>12 (11-15)</td>
<td>11 (10-13)</td>
</tr>
<tr>
<td>CCL2 (I)</td>
<td>(ng/ml)</td>
<td>0.4 (0.3-0.5)</td>
<td>0.3 (0.2-0.4)</td>
</tr>
<tr>
<td>CCL3 (I)</td>
<td>(pg/ml)</td>
<td>32 (31-35)</td>
<td>30 (29-31)</td>
</tr>
<tr>
<td>CCL3 (IV)</td>
<td>(pg/ml)</td>
<td>20 (18-22)</td>
<td>20 (18-20)</td>
</tr>
<tr>
<td>CCL4 (IV)</td>
<td>(pg/ml)</td>
<td>92 (61-180)</td>
<td>70 (65-87)</td>
</tr>
<tr>
<td>CCL20 (IV)</td>
<td>(pg/ml)</td>
<td>17 (8.7-37)</td>
<td>11 (7.4-14)</td>
</tr>
<tr>
<td>MMP9/NGAL (III)</td>
<td>(ng/ml)</td>
<td>31 (17-43)**</td>
<td>7.7 (4.4-15)</td>
</tr>
</tbody>
</table>

Table 4. The concentrations of inflammatory markers in exudates and serum. * P<0.05, ** P<0.01, *** P<0.005 between patients with CAD and healthy subjects.
4.2 MONOCYTE EXTRAVASATION AND EXPRESSION OF CD11B (I)

Monocytes are the dominating cell type that extravasate into the atherosclerotic plaque. The main objective in this paper was to evaluate if patients with CAD had an altered extravasation of monocytes. The expression and mobilization of adhesion molecules and chemokines related to monocyte extravasation were studied.

Patients with CAD had similar numbers of monocytes as healthy controls in both blood and chamber exudates. This indicates that there is no enhanced extravasation of monocytes during stable CAD. This is in line with the expression of adhesion molecules on circulating monocytes and the ratio of chemokines that did not differ between patients and controls. The data is further supported by similar in vitro transmigration over laminin coated inserts. Median transmigration was 13 (10-19) % in patients and 8 (6-23) % in controls and did not differ between the groups. Altogether, this indicates that stable CAD patients have a comparable monocyte extravasation into local inflammatory sites as healthy subjects.

4.2.1 Expression of CD11b

CD11b is important during inflammation since it binds to ICAM-1 on activated endothelium and to extracellular matrix. In contrast to stable CAD, acute phases of CAD are associated with an increased expression of CD11b on circulating monocytes [Meisel et al 1998, Lindmark et al 2001]. In the present study, the expression of CD11b on circulating monocytes did not differ between the groups (figure 8), which confirms that the patients were in a stable state of CAD. Acute CAD is also associated with increased serum levels of inflammatory markers [Luo et al 2004, Mizia-Stec et al 2003]. The similar concentrations of CRP, IL-6 and TNF-α in the patients compared to controls further indicate a stable disease and are likely related to interventions with statins [Luo et al 2004].

Figure 8. Basal expression of CD11b on peripheral and transmigrated cells (A). CD11b mobilization after stimulation with fMLP (B). P=0.002 in the un-stimulated blister between patients and controls (A). P=0.04 in the intermediately stimulated blister and P=0.006 the intensely stimulated blister following fMLP stimulation (B).
Despite similar expression of CD11b on circulating monocytes, patients with CAD had significantly lower expression of CD11b following extravasation. Figure 8 illustrates the basal expression of CD11b (figure 8A) and the expression of CD11b following fMLP stimulation (figure 8B). In order to discriminate the impact of extravasation, the expression of CD11b on extravasated monocytes was normalized to the expression on peripheral monocytes. The ratio of CD11b expression between extravasated monocytes and peripheral monocytes hence illustrates the mobilization of CD11b following extravasation. Table 5 illustrates the differences in CD11b ratio between patients and controls.

<table>
<thead>
<tr>
<th></th>
<th>Un-stimulated</th>
<th>significance</th>
<th>fMLP-stimulated</th>
<th>significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Un-stimulated blister</td>
<td>CAD = Healthy</td>
<td>0.051</td>
<td>CAD = Healthy</td>
<td>NS</td>
</tr>
<tr>
<td>Intermediately stimulated blister</td>
<td>CAD &lt; Healthy</td>
<td>0.04</td>
<td>CAD &lt; Healthy</td>
<td>0.008</td>
</tr>
<tr>
<td>Intensely stimulated blister</td>
<td>CAD &lt; Healthy</td>
<td>0.02</td>
<td>CAD &lt; Healthy</td>
<td>0.003</td>
</tr>
</tbody>
</table>

Table 5. Differences between patients with CAD and healthy subjects regarding the ratio of CD11b expression between extravasated monocytes and peripheral monocytes. Non-significant (NS).

The expressions of CD11b following extravasation to the stimulated chambers were altered in patients compared to healthy subjects. This was seen both in un-stimulated and the fMLP stimulated cells. No differences were seen on monocytes from the un-stimulated blister. This blister is incubated for 14 hours while the intermediately and intensely stimulated chambers are incubated for 10 hours. In addition, the inflammatory milieu in the stimulated chambers differs from the un-stimulated blister. While the chambers are stimulated with PBS or serum, the un-stimulated blister is a reaction to blister formation alone. Differences in the local inflammatory milieu as well as the duration of incubation are likely to modulate the expression of CD11b.

CD11b is localized in the plasma membrane and in granules that are mobilized following stimulation [Miller et al 1987]. Although the concentrations of inflammatory mediators were similar between the groups (table 4), one can not rule out that other factors, such as C5a, could be altered in patients with CAD. Heterologous desensitization could affect the lower expression of CD11b following fMLP stimulation but does not explain the reduced expression on un-stimulated cells. It is important to consider the effect of statins that reduce the expression of CD11b in hypercholesterolemic patients [Serrano et al 2001]. It is possible that the results are affected by medical interventions, but if this was the only explanation, a more homogenous reduction of CD11b would be expected. In addition, the concentrations of cholesterol in the blood did not correlate to CD11b expressions. The altered CD11b expression in the patients was neither an effect of an altered viability, as indicated by staining with annexin V and propidium iodide.

Human alveolar macrophages do not mobilize CD11b following fMLP or PMA stimulation [Miller et al 1987]. A possibility is therefore that extravasated monocytes
from patients with CAD have a more differentiated phenotype which reduce the mobilization of CD11b. The CD14+CD16+ monocytes are considered to be more differentiated, and an increased number of these cells in peripheral circulation is associated with CAD [Schlitt et al. 2004, Heine et al. 2008]. Markers related to monocyte maturation were further studied in paper II.

In order to evaluate if the chamber fluid influenced the expression of CD11b, leukocytes from healthy subjects were incubated with skin chamber fluid for two hours. Chamber fluid from patients with CAD did not alter the expression of CD11b compared to chamber fluid from healthy controls. The time point might have been too short and it is possible that leukocytes from patients with CAD might have responded differently. However, no direct influence on CD11b expression was detected on monocytes from healthy subjects.

The altered profile of CD11b in CAD is likely an integrated outcome from several factors. In order to determine if interaction with extracellular matrix could influence CD11b expression, in vitro extravasation was performed. Monocytes were induced to migrate over laminin coated membranes towards CCL2, since CD11b/CD18 is involved in this migration [Jiang et al. 1994]. The expression of CD11b on in vitro transmigrated monocytes was similar between patients and controls. Hence, interaction with laminin and in vitro migration towards CCL2 did not induce an altered expression of CD11b in patients with CAD compared to healthy subjects.

The concentration of proteases in the skin chamber exudate is not fully known but they may modulate the expression of CD11b. MMP-9/NGAL was increased in the immediately stimulated chamber exudate in patients with CAD compared to healthy controls (table 5) which could indicate increased proteolysis in CAD. Proteolytic degradation of CD11b following a strong stimulation has been demonstrated [Davey et al. 2000]. It is possible that cleavage of CD11b contributes to the lower expression of CD11b on extravasated neutrophils in patients with CAD.

Heparin is used as an anticoagulant in the skin chambers and this may influence the expression of CD11b since heparin is a ligand for CD11b/CD18 [Takada 2007]. The expression of CD11b was therefore analyzed in the presence of heparin, at a similar concentration as in the skin chamber. The results indicated no influence of heparin on CD11b expression neither in un-stimulated nor in fMLP stimulated cells.

Although the mechanism behind the observed dysregulation of CD11b in CAD is not known at present, it might have important biological consequences. During resolution of inflammation, monocytes emigrate from the inflammatory locus [Randolph 2008], and this characterizes plaque regression [Llodra et al. 2004]. The process of reverse migration is mediated by ICAM-1 and β2 integrins [Randolph and Furie 1996]. A lower expression of CD11b following extravasation might therefore contribute to monocyte entrapment at local inflammatory sites.
4.2.2 Expression of VLA-4

VLA-4 is also important for monocyte extravasation during inflammatory conditions. The expression of VLA-4 on peripheral monocytes is slightly increased during acute CAD [Meisel et al. 1998]. Our results indicated no significant difference in VLA-4 between patients and controls on either peripheral or extravasated monocytes. The expression of VLA-4 was decreased following extravasation, which might be an effect of shedding. Shedding of LFA-1 in a blister model concomitant with a non-significant reduction in VLA-4 has been reported [Evans et al. 2006].

4.3 THE PHENOTYPE OF EXTRAVASATED MONOCYTES (II)

The main objective in this paper was to evaluate if extravasated monocytes were functionally different in patients with CAD compared to healthy controls. Markers associated with the role of monocytes/macrophages in CAD were analyzed. Co-expression of CD14 and CD16 was studied since the CD14+CD16+ monocytes are associated with CAD. Further assessments included expression of CX3CR1 that mediates extravasation of CD14+CD16+ monocytes, CD86 and HLA-DR that regulate antigen presentation, CD36 that is deeply involved in foam cell formation, and the binding of acLDL.

4.3.1 Accumulation of CD14+CD16+ monocytes

The number of circulating CD14+CD16+ monocytes correlates to the thickness of the carotid intima media [Rogacev et al. 2009] and the prevalence of CAD [Schlitt et al. 2004, Heine et al. 2008]. In our study, patients with CAD had similar numbers of CD14+CD16+ monocyte compared to healthy controls in both blood and chamber exudate. This could reflect that the patients were in a stable phase and under the influence of medical interventions.

The median percentage of CD14+CD16+ monocytes in the chamber exudates was 40% compared to 10% in peripheral blood. This could indicate either accumulation of CD16+ monocytes or induction of CD16 by local factors in the skin chamber and by adhesive interactions during extravasation. The expression of CD16 can be induced by in vitro incubation with M-CSF or GM-CSF in presence of IL-4 and IL-10 [Li et al. 2004, Allavena et al. 1998]. In addition, P-selectin has been shown to induce maturation of CD16 positive dendritic-like cells [Li et al. 2003]. Maturation of CD16 positive cells in vitro is vastly influenced by the specific settings and does not truly reflect formation of the CD16+ subtype in vivo. CD14+CD16+ monocytes from peripheral blood have a genotype and a phenotype that partly resembles those in both macrophages and dendritic cells [Ancuta et al. 2009]. Hence, the CD14+CD16+ subtype is considered to be more mature.

Significantly increased concentrations of IL-10 (table 4) and GM-CSF [Kuhns et al. 1992] have previously been detected in chamber exudate. We therefore tested whether the chamber fluid could induce the expression of CD16 on peripheral monocytes. CD16 was not induced following 18 hours of incubation, favoring the idea that adhesion-mediated events might be crucial in the induction of CD16. Alternatively,
CD16 positive monocytes are selectively recruited to the skin chamber. Furthermore, it can not be ruled out that the CD14+CD16+ monocytes have an altered emigration from the skin chamber, favoring accumulation of this subtype.

4.3.2 Expression of CX₃CR1

The recruitment of CD14+CD16+ monocytes to inflammatory sites is particularly associated with fractalkine. Figure 9 views the expression of CX₃CR1, CD86, HLA-DR and CD36 on monocytes from peripheral blood and skin chamber exudate.

Figure 9. The expressions of CX₃CR1, CD86, HLA-DR and CD36 assessed by flow cytometry. The cytograms view the binding of the isotype and the specific antibody. The median MFI for patients and controls are indicated to the right of each cytogram. No significant differences were detected between patients and controls.
More than 95% of the circulating monocytes expressed the fractalkine receptor CX3CR1. In agreement with a previous publication, the expression of CX3CR1 was similar between CAD patients and healthy subjects [Damas et al 2005]. Following extravasation, the expression of CX3CR1 was significantly reduced, which may indicate cleavage during extravasation. A lower expression of CX3CR1 on lymphocytes from synovial fluid compared to peripheral blood has previously been demonstrated [Ruth et al 2001] and may indicate a general down-regulation of CX3CR1 following extravasation.

### 4.3.3 Markers associated with antigen presentation

The expression of HLA-DR and the co-stimulatory molecule CD86 increased following extravasation (Figure 9). HLA-DR is associated with maturation of mononuclear cells into antigen-presenting cells. In order to determine if the skin chamber exudate could influence the expression of HLA-DR, in vitro incubations were performed. Incubation of peripheral monocytes with chamber exudate did not induce HLA-DR. CD16+ monocytes have an increased presentation of antigens that coincide with increased expressions of HLA-DR and CD86 [Skrzeczynska-Moncznik et al 2008]. The higher expression of HLA-DR on extravasated monocytes may therefore be closely linked to accumulation of CD16+ monocytes. However, this can only be speculated upon, since multicolor analysis of CD14, CD16 and HLA-DR was not performed.

![Figure 10. The Expression of CD86 on monocytes from peripheral blood and chamber exudate. The expression was increased following extravasation and could be further induced by stimulation with fMLP. No significant differences were detected between patients and controls. Filled triangles refer to CAD patients and open triangles refer to healthy controls.](image)

The expression of CD86 was higher on extravasated monocytes compared to circulating monocytes and could be further induced by in vitro stimulation with fMLP, indicating a pool of mobilized CD86 (Figure 10). The increased expression of CD86 following in vitro fMLP stimulation may also suggest a linkage between fMLP mediated innate and adaptive immune responses. CD16+ monocytes have an increased
expression of CD86 [Skrzeczynska-Moncznik et al 2008] which may contribute to the higher expression of CD86 on exudated monocytes.

An increased expression of CD86 has been detected on monocyte derived dendritic cells from patients with CAD compared to healthy controls [Dopheide et al 2007]. However, these cells were cultivated in vitro for nine days. Our results suggest that there is no direct alteration in CD86 expression following extravasation in patients with CAD compared to healthy controls. It is however possible that changes between patients with CAD and healthy controls may commence following differentiation and maturation of antigen-presenting cells.

### 4.3.4 Expression of CD36 and binding of acLDL

CD36 is the main receptor for oxLDL and highly involved in foam cell formation [Endemann et al 1993]. The expression of CD36 is induced during differentiation [Huh et al 1996] and following adhesion to TNF-activated endothelial cells [Huh et al 1995]. A modest increase in the expression of CD36 was detected on extravasated monocytes compared to circulating monocytes (figures 9 and 11) which may account for interactions during extravasation as well as maturation. There was no difference between patients and controls. The expression of CD36 is induced by both naïve and modified LDL [Han et al 1997]. It can only be speculated on if the reduced concentration of plasma LDL in the patients influenced the expression of CD36. In addition, one can not rule out cleavage of CD36, since an elevated concentration of soluble CD36 is detected in patients with type 2 diabetes [Handberg et al 2006].

![Figure 11. The expression of CD36 on monocytes from peripheral blood and chamber exudate. The expression was increased following extravasation and could be further induced by stimulation with fMLP. No significant differences were detected between patients and controls. Filled triangles refer to CAD patients and open triangles refer to healthy controls.](image)
The expression of CD36 on extravasated monocytes was further induced by in \textit{vitro} stimulation with fMLP (Fig 11). CD36 binds to thrombospondin-1 and increased thrombospondin binding that coincides with degranulation is detected on stimulated human neutrophils [Suchard \textit{et al} 1992]. Together, this might indicate an intracellular pool of CD36 that can be mobilized.

The expression of CD36 following fMLP stimulation might be influenced by monocyte/platelet aggregates. Mononuclear cells were therefore separated over ficoll and then subjected to repeated low g-force washes prior to fMLP stimulation. This method has previously been shown to markedly reduce the number of platelets. The results indicated that platelets had no influence on the expression of CD36.

The expression of CD36 was further assessed following \textit{in vitro} incubation with chamber fluid. The results were normalized against the expression of CD36 on monocytes that were incubated with cell medium alone. Blister fluid from healthy subjects did not alter the expression of CD36, the ratio to un-stimulated cells was 0.98 (0.79-1.16). Chamber fluid from patients with CAD significantly induced the expression of CD36. The median ratio between chamber fluid from CAD patients and cell medium was 1.25 (1.16-1.36). This indicates that the inflammatory milieu in patients with CAD might influence the expression of CD36. Factors in the skin chamber fluid that could induce CD36 include M-CSF and IL-4 [Yesner \textit{et al} 1996].

The binding of modified LDL was assessed by flow cytometry on cells that had been incubated with acLDL conjugated to a fluorescent dye. The binding of acLDL was significantly higher on extravasated cells (20 (17-24) MFI) compared to cells from peripheral circulation (8.8 (7.8-10) MFI) in healthy subjects. No significant differences were detected between patients and controls. Binding of acLDL can be mediated by CD36 as well as SR-A1 [Kunjathoor \textit{et al} 2002]. The expression of SR-A1 was assessed by flow cytometry. The expression of SR-A1 was comparable in blood (29.4 (23.9-35.1) %) and chamber exudate (29.3 (17.45-39.45) %) in healthy controls and did not differ to patients. The higher proportion of CD16\textsuperscript{+} monocytes in the exudate compared to in blood might affect the results on SR-A1 since CD16\textsuperscript{+} monocytes have a lower expression of SR-A1 [Draude \textit{et al} 1999]. However, the increased expression of CD36 following extravasation might associate with the enhanced binding of modified LDL.

4.4 \textbf{THE FUNCTION OF EXTRAVASATED NEUTROPHILS (III)}

The objective was to study activation of circulating and extravasated neutrophils. Soluble markers associated with neutrophil activation, IL-8 and MMP9/NGAL, were measured in serum and chamber exudates. Expression of CD11b and production of ROS were assessed as markers of functional-related activation.

A high number of neutrophils is associated with an increased risk and poor outcome of CAD [Horne \textit{et al} 2005, Haumer \textit{et al} 2005]. Furthermore, the number of neutrophils correlates to the concentration of pro-inflammatory molecules in circulation [Nijm \textit{et al} ...]
In the present study, similar numbers of neutrophils and concentrations of pro-inflammatory markers IL-6 and CRP were detected in patients with CAD compared to healthy controls. This may indicate that the patients were in a stable disease and in addition treated with statins that are endowed with anti-inflammatory effects [Luo et al 2004].

4.4.1 Concentration of IL-8 and MMP-9/NGAL

Markers associated with neutrophil activation, IL-8 and MMP9/NGAL, were significantly higher in serum from patients compared to healthy controls (table 4). This could indicate that circulating neutrophils are primed. An increased concentration of IL-8 in plasma has previously been reported in both stable and unstable CAD [Hashmi and Zeng 2006]. Furthermore, IL-8 is an independent predictor of disease outcome [Inoue et al 2008]. The increased concentrations of IL-8 and MMP-9/NGAL in CAD might be an integrated outcome of several factors. IL-8 is induced in vascular smooth muscle cells and endothelial cells following activation with IL-1β [Wang et al 1991]. In addition, shear stress induces the production of IL-8 and MMP-9 in endothelial cells [Cheng et al 2008, Sun et al 2007].

The expression of MMP-9/NGAL could be influenced by the release of gelatinase and specific granules [Kjeldsen et al 1994 a, and b]. NGAL is associated with neutrophil specific granules [Kjeldsen et al 1994 a] and extravasation to the skin chamber has previously been shown to mobilize 20% of the specific granules [Sengelov et al 1995]. However, the concentration of MMP-9/NGAL is not necessary linked to neutrophil degranulation or the concentration of individual components since most NGAL form monomers or dimmers that are not associated with MMP-9 [Kjeldsen et al 1993]. NGAL protects MMP-9 from degradation and an increased concentration of the complex could contribute to an increased activity of MMP-9 in CAD [Yan et al 2001]. A positive feedback between IL-8 induced release of MMP-9 and MMP-9 induced truncation of IL-8 exists, and truncated IL-8, compared to non-truncated, has an increased biological activity [Van den Steen et al 2000]. MMP-9 has been attributed to weakening of the fibrous cap and is a predictor of CAD outcome [Blankenberg et al 2003]. The concentration of MMP-9 is increased in patients with stable as well as acute CAD [Zeng et al 2005].

4.4.2 Expression of CD11b and production of ROS

The expression of CD11b and production of ROS were assessed to study neutrophil function (Table 6). Basal expression of CD11b and production of H₂O₂ were similar between patients and controls in both blood and exudated neutrophils. Following in vitro stimulation, extravasated neutrophils from the intensely stimulated chamber in patients with CAD had a significantly lower expression of CD11b and a lower production of H₂O₂ compared to healthy controls (table 6). A general trend towards a lower response following extravasation and concomitant in vitro stimulation was seen in the patients but was only significant in exudate from the intensely stimulated chamber.
Un-stimulated | In vitro stimulated
---|---
| CAD | Healthy | CAD | Healthy

**Expression of CD11b (MFI)**

<table>
<thead>
<tr>
<th></th>
<th>Blood</th>
<th>Intermediately stimulated chamber</th>
<th>Intensely stimulated chamber</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.3 (3.4-6.8)</td>
<td>53 (47-59)</td>
<td>53 (43-56)</td>
</tr>
<tr>
<td>CAD</td>
<td>4.7 (3.6-5.4)</td>
<td>62 (50-79)</td>
<td>67 (53-75)</td>
</tr>
<tr>
<td>Healthy</td>
<td>81 (72-99)</td>
<td>113 (105-132)</td>
<td>99 (79-120)**</td>
</tr>
<tr>
<td></td>
<td>85(75-110)</td>
<td>138 (110-172)</td>
<td>118 (105-143)</td>
</tr>
</tbody>
</table>

**Production of H₂O₂ (MFI)**

<table>
<thead>
<tr>
<th></th>
<th>Blood</th>
<th>Intermediately stimulated chamber</th>
<th>Intensely stimulated chamber</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.4 (2.9-8.1)</td>
<td>6.6 (5.7-9.3)</td>
<td>4.6 (2.0-9.7)</td>
</tr>
<tr>
<td>CAD</td>
<td>3.1 (1.9-6.3)</td>
<td>7.0 (5.5-10.7)</td>
<td>7.5 (5.0-8.7)</td>
</tr>
<tr>
<td>Healthy</td>
<td>143 (106-189)</td>
<td>90 (57-116)</td>
<td>94 (38-123)*</td>
</tr>
<tr>
<td></td>
<td>188 (165-222)</td>
<td>139 (89-157)</td>
<td>128 (95-199)</td>
</tr>
</tbody>
</table>

Table 6. The expression of CD11b and production of ROS in neutrophils from peripheral blood and chamber exudate. fMLP was used as an stimuli for assessment of CD11b expression and PMA was used as a stimuli for assessment of ROS production. * P<0.05 and ** P<0.01 between patients and controls.

Total and high affinity CD11b expressions on neutrophils from peripheral blood is similar in patients with stable CAD compared to healthy controls [Lindmark et al 2001, Sarndahl et al 2007]. This is in agreement with our results on un-stimulated peripheral and exudated neutrophils.

An increased production of ROS in circulating neutrophils from patients with acute CAD has been reported following PMA stimulation [Takeshita et al 1997]. On the contrary, a reduced production of ROS has been reported during stable CAD following stimulation with C3bi opsonised yeast [Sarndahl et al 2007]. In our study, there was a trend towards a lower production of ROS in circulating neutrophils from patients with CAD compared to healthy controls, however this was not significant. Possible discrepancies may be explained by differences in stimuli, duration and choice of measurement.

The main reservoir of fMLP receptors is localized in the easily mobilized secretory vesicles [Sengelov et al 1994] that also contain CR1. In order to test if the differences were induced by a reduced mobilization of secretory vesicles, CR1 was analyzed. The expression of CR1 on circulating neutrophils was 32 (24-46) % in patients and 27 (13-54) % in controls. Corresponding numbers on extravasated neutrophils was 99 (95-100) % and 99 (97-100) %. Thus, the expression of CD11b in patients with CAD is not explained by an altered mobilization of fMLP receptors. CD11b is mainly localized to specific and gelatinase granules [Sengelov et al 1993]. The specific granules are also a reservoir of the membrane bound part of the NADPH [Borregaard et al 1983]. The expression of CD11b on un-stimulated and stimulated neutrophils from blood indicates
similar mobilization of specific and gelatinase granules between patients and controls. Therefore, the observed differences in CD11b and H$_2$O$_2$ between patients and controls are likely not an effect of altered granule mobilization.

A potential priming of circulating neutrophils favors the hypothesis that patients with CAD should have an increased expression of CD11b and an increased production of H$_2$O$_2$ following activation. However, neutrophils can be primed in several ways: in circulation by an increased concentration of IL-8, during extravasation due to integrin mediated activation and in the chamber exudate by inflammatory mediators. IL-8 priming of fMLP induced oxidative burst is observed in vitro [Guichard et al 2005]. However, the mechanism of chronic exposure to IL-8 is not known and could also result in desensitization.

Following excessive stimulations with repeated stimuli that utilize similar intracellular pathways, neutrophils become desensitized. An important inflammatory mediator in the skin chamber is C5a [Follin et al 1991]. Heterologous desensitization between IL-8, C5a and fMLP has been reported [Richardson et al 1995, Tomhave et al 1994]. In addition, desensitization between IL-8 and the β$_1$-integrin ligand fibronectin has been reported [Stanton et al 1999]. Desensitization may hence occur at many different levels. PMA is a receptor independent stimulus that directly activates PKC. The fMLP receptor is however not desensitized by PKC mediated phosphorylation [Richardson et al 1995]. The observed response could be mediated by desensitization below PKC at the level of PLC or further downstream.

The expression of CD11b on circulating neutrophils from patients with stable CAD has been studied following stimulation with IL-8 or LTB$_4$ [Sarndahl et al 2007]. The former study did not indicate an altered response in patients with CAD. However, IL-8 and LTB$_4$ are less potent than fMLP. A hierarchy between different attractants exists with fMLP being an end target [Campbell et al 1997, Heit et al 2002]. The lower expression of CD11b in our study was only significant in extravasated neutrophils that were further stimulated in vitro. Desensitization is expected to be most pronounced following strong stimulation, such as stimulation of extravasated cells.

An additional mechanism for the reduced expression of CD11b in patients with CAD could be proteolytic shedding, which has previously been demonstrated following intense stimulation [Davey et al 2000]. Leukocyte activation is influenced by the composition of lipid rafts in the cellular membrane. One possible explanation is that statin-treated patients have an altered signaling due to changes in the lipid rafts. The importance of lipid rafts for activation and exocytosis has been reported in natural killer cells [Inoue et al 2002]. In addition, neutrophils from patients with myelodysplasia (a hematological disorder) have a reduced production of ROS that coincides with an impaired formation of lipid rafts [Fuhler et al 2007].
The objective in this paper was to study if extravasated neutrophils could have an immuno-modulatory role at a local inflammatory site. Gene expression in extravasated neutrophils and the potential impact of IL-1 activation were assessed.

The number of circulating neutrophils was significantly increased in patients with CAD 3.8 (3.0-3.9) x10^9 neutrophils/μL compared to in healthy controls 2.6 (1.8-3.0) x10^9 neutrophils/μL. This finding is in line with previous studies that have shown a correlation between the number of circulating neutrophils and risk of CAD [Horne et al 2005, Haumer et al 2005].

Neutrophils are considered to be fully mature when they leave the bone marrow and are hence transcriptionally silent. However, induction of gene transcription occurs following neutrophil extravasation. Gene expression analysis of neutrophils from a chamber exudate has previously shown an induction of IL-1, NFκB, CCL3 and CXCL2, among others [Theilgaard-Monch et al 2004]. In addition, extravasated neutrophils from an endotoxin-induced inflammation in the lung have an induced expression of IL-1, IL-8, CCL2, CCL3 and CCL4, among others [Coldren et al 2006]. Corresponding genes are also induced following exposure to lipopolysaccharide [Malcolm et al 2003].

The expression of genes in purified neutrophils from peripheral blood and chamber exudates was studied with microarray, assessing 20x10^3 genes. Pathway analysis indicated a general induction in the IL-1 axis following extravasation. Induction of IL-1R1, IL-1RN, NFκB, CCL3, CCL4, CCL20 and CXCL2 was further confirmed by RT-PCR (Figure 12A). These findings are in line with previous publications on extravasated neutrophils [Theilgaard-Monch et al 2004, Coldren et al 2006].

Figure 12. Gene expression analyzed by gene array (n=3+3) and RT-PCR (n=7+7). Changes in gene expression between extravasated and peripheral neutrophils (A). Changes between patients with CAD and healthy controls (B). Interleukin-1 receptor-associated kinase 1 (IRAK1).

IL-1 is essential for activation of endothelial cells and production of chemokines at local inflammatory sites [Pober and Sessa 2007] and an induced migration of
neutrophils has been demonstrated following IL-1 activation of endothelial cells [Furie and McHugh 1989]. Local IL-1 is also an important factor for leukocyte activation, IL-1 primes neutrophils for IL-8 induced responses [Brandolini et al 1996] and mediates a weak activation of NFκB in circulating neutrophils [McDonald et al 1997]. Stronger stimuli such as TNF-α and LPS have a greater impact on NFκB activation [McDonald et al 1997] and mediate induction of chemokines and cytokines [Cloutier et al 2007].

In vitro incubation of purified neutrophils from the blood of healthy subjects confirmed the induction of CCL3, CCL4, CCL20, CXCL2 and NFκB genes by IL-1. Expressions of CCL3 and CCL4 were further assessed by immunoassay on the supernatant from IL-1 stimulated neutrophils. IL-1 induced a low production and release of both chemokines. Our data indicate that IL-1 may activate neutrophil gene transcription and that IL-1 stimulated neutrophils constitute a source of chemokines.

In order to determine a potential role for the IL-1 axis in extravasated neutrophils, the expression of IL-1R1 was measured by flow cytometry and immuno electron microscopy. Figure 13 views the expression of IL-1R1 on neutrophils from peripheral blood and the chamber exudate.

![Figure 13. Expression of IL-1R1 assessed by flow cytometry. The expression of IL-1R1 was significantly increased following extravasation and could be induced by fMLP stimulation in both healthy controls and patients with CAD.](image)

The expression of IL-1R1 on circulating neutrophils was low and could be significantly induced by fMLP. Extravasated neutrophils had an increased expression of IL-1R1 compared to circulating neutrophils and the expression could be further induced by fMLP. Immuno electron microscopy indicated an intracellular pool of IL-1R1. The expression of IL-1R1 on human neutrophils from peripheral blood has been estimated to 900 receptors per cell and internalization of radiolabeled IL-1 indicate that the receptors are functionally active [Parker et al 1989]. It is intriguing to speculate if the increased expression of IL-1R1 on extravasated neutrophils represents newly synthesized receptors. An increased gene expression of IL-1R1 was detected following extravasation. In addition, a higher number of IL-1R1 was detected in extravasated neutrophils compared to in circulating neutrophils assessed by immuno electron microscopy.
microscopy. The intracellular location and induction of IL-1R1 following fMLP stimulation might indicate a pool of IL-1R1 that can be mobilized. However, soluble IL-1R1 has been demonstrated in serum and at an inflammatory location [Okamoto et al 2009, Arend et al 1994]. Whether the intracellular pool of IL-1R1 represents soluble receptors that have been phagocytosed is not known at present. The expression of soluble IL-1R1 in the chamber exudate was not measured. However, the concentration of soluble IL-1R1II was slightly increased in the chamber exudate compared to in serum in both patients and healthy controls (table 4). Whether IL-1R1 follows the same expression profile as IL-1R1II in the blister fluid can only be speculated on. Thus, the induced expression of IL-1R1 following fMLP stimulation might indicate the existence of mobilized IL-1R1.

The concentrations of IL-1α, IL-1β and IL-1Ra were increased in the chamber exudate compared to in serum in both patients and controls (table 4). Taken together, these results indicate that the IL-1 axis is activated following extravasation in both CAD patients and healthy subjects. Increased gene and protein expressions of IL-1β, IL-1R1 and IL-1Ra have been detected in specimens from atherosclerotic arteries [Olofsson et al 2009]. In addition, polymorphism in IL-1Ra is associated with CAD [Olofsson et al 2009]. Circulating mononuclear cells from patients with stable and acute CAD have increased expressions of IL-1α, IL-1β and IL-1Ra that are modulated by statins [Weahre et al 2004]. Similar concentrations of IL-1α, IL-1β, IL-1Ra and soluble IL-1R1II were detected between patients and controls (table 4) and might be the result of medical interventions.

Minor differences in gene expression were noted in peripheral blood from patients with CAD, compared to healthy controls, 86 genes had an increased expression and 131 genes had a lower expression. Following extravasation, there was a higher expression of 400 genes and a lower expression of 1525 genes in patients compared to healthy controls. Pathway analysis indicated differences in the IL-1 axis in patients with CAD compared to healthy controls (Figure 14). The increased expression of CCL20 and CXCL2 in extravasated neutrophils from patients with CAD compared to healthy controls was confirmed by RT-PCR (Figure 12B).

An increased expression of CCL20 has been demonstrated in extravasated neutrophils from synovial fluid of patients with rheumatoid arthritis [Schlenk et al 2005]. CCL20 binds to CCR6 that is mainly expressed on dendritic cells and activated T- and B-lymphocytes [Baba et al 1997]. An increased production of CCL20 in CAD could be important for recruitment of these cells. The expression of CD1 [M elian et al 1999] and co-stimulatory molecules [de Boer et al 1997] in human atherosclerotic plaques indicate that antigen-specific T-lymphocytes might be involved in the inflammatory reaction.

Chemokines in the CXCL family are generally considered to activate neutrophils. However, CXCL2 mediates P-selectin dependent arrest of monocytes under flow [Smith et al 2005]. Furthermore, CXCR2, the receptor of CXCL2, is induced on monocytes by oxLDL [Lei et al 2002] and expression of CXCR2 is detected in human atherosclerotic lesions [Boisvert et al 1998]. Furthermore, studies in knock-out mice indicate that CXCR2 is important for macrophage accumulation and plaque progression.
An increased expression of CXCL2 at local inflammatory sites may therefore have an important function in accumulation of both neutrophils and monocytes.

The concentrations of CCL3, CCL4 and CCL20 were significantly higher in the exudate compared to in serum in both patients and controls (table 4). No significant differences were detected between patient and controls. The inflammatory milieu in the skin chambers is determined by the combined production of cytokines and chemokines from infiltrated leukocytes, endothelial cells and fibroblasts. Altered production from individual cell types might therefore be masked. In addition, statins reduce the expression and spontaneous release of chemokines [Waehre et al 2003, Veillard et al 2006]. This may be reasons for discrepancies between gene and protein expressions.

![Figure 14. Changes in gene expression (>1.5x) in extravasated neutrophils from patients with CAD compared to healthy controls. Data was analyzed by the ingenuity software according to biological function. Red indicates an increased and green a lower expression in patients with CAD compared to healthy controls.](image-url)
CONCLUSIONS

I. Patients with CAD had similar numbers of extravasated monocytes at the local inflammatory sites as healthy controls. However, compared to healthy subjects, extravasated monocytes from patients with CAD had a lower expression of the adhesion molecule CD11b. This may contribute to entrapment of monocytes at local inflammatory sites.

II. A higher percentage of CD14+CD16+ monocytes was observed in the skin chamber compared to in blood which might indicate accumulation of more mature cells. In addition, extravasated monocytes had increased expressions of markers associated with antigen presentation and foam cell formation. No differences were detected between patients and controls. However, the skin chamber fluid from patients with CAD induced the expression of CD36, favoring the concept of a pro-atherosclerotic local milieu in patients with CAD.

III. An increased expression of IL-8 was detected in serum from patients with CAD and might associate with peripheral priming of the neutrophils. The concentration of MMP-9/NGAL was increased in the circulation and at the local inflammatory site and this could contribute to the pathalogy in CAD. The responsiveness of extravasated neutrophils from patients with CAD was reduced compared to healthy controls. This might indicate desensitization and an altered intracellular activation in extravasated neutrophils in patients with CAD.

IV. Extravasated neutrophils had an increased expression of chemokines, and pathway analysis indicated activation of the IL-1 axis. The expression of IL-1R1 increased following extravasation, and in vitro stimulation with IL-1 resulted in expression of chemokines. Patients with CAD had increased expressions of CCL20 and CXCL2 following extravasation which could contribute to accumulation of mononuclear cells at a local inflammatory site. Hence, extravasated neutrophils may enhance the pro-inflammatory milieu.
Several hypotheses have been highlighted in this thesis and with them, many more questions have followed. I would like to emphasize a few of those and the future perspectives that have arisen during this time.

The transmigration process is highly regulated at several different levels. Adhesion molecules are regulated by the avidity and the affinity and chemokines are regulated by the formation of gradients, truncations and by dimerisation. Although the high affinity conformation of CD11b did not differ on circulating neutrophils during CAD [Sarndahl et al 2007], it is appealing to investigate the expression following extravasation. The affinity of CD11b is regulated during transmigration by inside out signals and one approach is to measure the active epitope of CD11b in relation to total CD11b expression following extravasation. This could give additional information on adhesion molecule regulation in CAD.

In addition, the increased concentration of proteases in the local inflammatory milieu in patients with CAD could present a possible mechanism for the truncation of chemokines. Since many truncated chemokines have an increased biological activity, it would be interesting to examine if patients with CAD have more truncated chemokines compared to healthy subjects. In addition, it would be of interest to examine if truncated chemokines at local inflammatory sites interfere with the balance of extravasation and emigration and hence could contribute to leukocyte entrapment.

The skin blister fluid from patients with CAD induced the expression of CD36. Additional experiments are needed to explore if patients with CAD have an increased concentration of local factors with pro-atherosclerotic functions. This hypothesis needs to be further tested by incubating extravasated monocytes with their own chamber fluid in vitro. This enables a continued differentiation and discrimination of functional alterations in patients with CAD and healthy controls. Also, the potential factors in the skin chamber fluid needs to be identified. These include cytokines associated with differentiation such as IL-4 and GM-CSF.

Furthermore, the increased proportion of CD14+CD16+ monocytes at the local inflammatory site compared to in blood suggests a potential different role of this subtype compared to the CD14+CD16- monocytes at an inflammatory location. It is not know today if the CD14+CD16+ monocytes have an increased migration to the skin chamber or if CD16 is induced following extravasation. The skin blister fluid did not influence the expression of CD16 but other mechanisms such as adhesion mediated activation could have potential effects [Li et al 2003]. The expression of CD16 could be examined following transwell mediated migration in vitro, following cross-linking of adhesion molecules and after incubation with activated endothelial cells.

Neutrophils from patients with CAD appear to have an altered activation profile. It would therefore be of interest to further evaluate activation of peripheral and extravasated neutrophils in patients with CAD and healthy subjects. Potential targets of
exploration include the composition of lipid rafts, PCK, PLC, Ca$^{2+}$ mobilization and tyrosine phosphorylation.

Additional information is also needed on the expression of IL-1R1 following extravasation. The concentration of soluble IL-1R1 needs to be determined and the potential impact on phagocytosis has to be unraveled. The location of IL-1R1 in intracellular organelles and the increased surface expression following stimulation suggests IL-1R1 mobilization, however, this needs further studies.

Data from patients on medical interventions must always be thoroughly scrutinized. To include patients with limited interventions would of course be preferable but does not reflect the clinical reality. Many of the observed results could be influenced by statins which are endowed with several anti-inflammatory effects. In vitro experiments in the presence of statins are possible and could provide additional information of the potential impact of statins. However, a major obstacle that has to be solved is the hydroxylation and production of the active metabolite. Statins are processed by the liver into active metabolites and to incubate cells with intact statins would not truly reflect the biological activity.

In the best of world it would also be interesting to incubate the skin chamber over a longer period of time to allow potential modulation of the inflammatory milieu by extravasated neutrophils. In addition, if there were plenty of cells, co-cultures with extravasated monocytes and lymphocytes would be appealing to study.
SAMMANFATTNING PÅ SVENSKA


I denna avhandling har vi använt en hudkammarmodell som gör det möjligt att studera celler som har transmigrerat från blodet till en lokal inflammation. Hudblåsor induceras på underarmen genom sugkoppar. Denna process lyfter det översta hudlagret så att det kan avlägsnas. Plastkoppar monteras sedan över såret och fylls med en buffertlösning eller serum. På detta sätt induceras en lokal inflammation i direkt anslutning till hudens kapillärbädd vilket möjliggör skördning av transmigrerade celler.

Syftet med avhandlingen var att studera transmigrerade monocyter och neutrofiler i patienter med kranskärlssjukdom och friska kontroller. Markörer kopplade till transmigrationsprocessen och till olika cellspecifika funktioner vid arterioskleros analyserades. Samtliga patienter hade haft en akut manifestation av sin sjukdom men var vid försöket stillfället stabila och utan tecken på andra inflammatoriska tillstånd.


Vi har i denna avhandling påvisat en möjlig bidragande faktor till att monocyter ansamlas i arteriosklerotisk vävnad. Detta visades dels genom ett lägre uttryck av adhesionsmolekylen CD11b och dels genom att transmigrerade neutrofiler från patienter med kraniskärllssjukdom hade en ökad produktion av vissa monocyctrelaterade kemokiner. Vi har även påvisat att neutrofiler från patienter med kraniskärllssjukdom har en förändrad aktivering och kan vara refraktära efter transmigration.
I would like to send my sincere gratitude to the following persons who have contributed to this thesis in so many different ways.

Joachim Lundahl, my main supervisor, thanks for believing in me and always pushing me one step forward. Your enthusiasm and endless ideas are the cornerstones of research, I appreciate your advises and new ways to scrutinize data. Finally, thanks for being such a friendly person.

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My co-authors: Ali Moshfegh, you are such a good friend, thanks for your support when life as a PhD-student was tough. Elham Dadfar, my former room mate and student companion, thanks for good cooperation. I will remember our travels; go west is imprinted in my memory of a nice day along the Australian coast.

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