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LEUKOCYTE TRANSMIGRATION AND GENE EXPRESSION IN HEALTHY SUBJECTS AND PATIENTS WITH RENAL FAILURE–APPLICATION OF THE SKIN CHAMBER TECHNIQUE

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Stockholm 2006

Published and printed by Karolinska University Press
Box 200, SE-171 77 Stockholm, Sweden

ISBN 91-7140-752-9

To my parents
and Ali

ABSTRACT

The migration of leukocytes from the peripheral circulation into infected or injured tissue is a fundamental step in the host-defense mechanism. The skin chamber technique is a well documented method for studies of leukocyte transmigration and function *in vivo*. Patients with chronic renal failure are highly susceptible to infections and one contributing factor is dysfunctional leukocytes.

The *aim* of this thesis was to analyze the transmigration and state of activity in terms of adhesion molecule expression of *in vivo* transmigrated neutrophils and monocytes in healthy subjects and patients with renal failure, using the skin chamber technique.

Results: We have shown that monocytes that have been newly recruited to sites of interstitial inflammation *in vivo*, prior to their differentiation to macrophages, have a preserved ability to respond to challenge with bacterial peptides in terms of CD11b upregulation and intracellular hydrogen peroxide production in healthy subjects. This indicates that newly recruited monocytes play an important role in the immediate response against invading pathogens. In order to study the immune response at the interstitial site in patients with renal failure, monocyte transmigration and state of activity in terms of CD11b expression was analysed in patients with moderate to severe renal failure, patients on peritoneal dialysis and healthy subjects. In addition to monocytes, we investigated granulocytes from patients on peritoneal dialysis.

Transmigrated monocytes from patients with severe renal failure had a reduced ability to upregulate CD11b at the interstitial site of inflammation compared with cells collected from healthy subjects. The reduced CD11b expression was more dependent on cellular factors than on the concentration of soluble mediators in the interstitial milieu. A reduced ability to upregulate CD11b was also observed in monocytes and neutrophils from patients on peritoneal dialysis. Since CD11b plays a crucial role for innate immunity to invading microbes, these phenotypic aberrations may have pathophysiological consequences in terms of increased susceptibility to infectious diseases, a phenomenon observed in patients with renal failure.

In order to understand the molecular mechanisms that contribute to the leukocyte dysfunction observed in patients with renal failure, gene expression profiling on peripheral and *in vivo* transmigrated neutrophils from patients with severe renal failure and healthy subjects was performed. Neutrophils from patients with renal failure showed a divergent gene expression pattern, compared to neutrophils from healthy subjects in the peripheral circulation and at the site of interstitial inflammation. The greatest differences were observed at the interstitial site. At that site, neutrophils from patients with renal failure had a higher gene expression of proinflammatory cytokines and cytokines involved in T-cell cell recruitment.

In conclusion, a gradual loss of renal function is associated with impaired leukocyte CD11b expression at the interstitial site of inflammation. This is partly improved by renal replacement therapy. Furthermore, *in vivo* transmigrated neutrophils from patients with renal failure have a more pronounced expression of proinflammatory genes compared to healthy subjects. Our findings contributes to a better understanding of factors involved in the higher rate of infections observed in patients with renal failure. These data may generate potential platform for new therapeutic interventions.

LIST OF PUBLICATIONS

I Dadfar E, Jacobson SH, Lundahl J
In vivo transmigrated human monocytes have a preserved responsiveness towards bacterial peptides in terms of CD11b up-regulation and intracellular hydrogen peroxide production.
Submitted

II Dadfar E, Lundahl J, Jacobson SH
Monocyte adhesion molecule expression in interstitial inflammation in patients with renal failure.
Nephrol Dial Transplant. 2004; 19(3):614-22.

III Dadfar E, Lundahl J, Fernvik E, Nopp A, Hylander B, Jacobson SH
Leukocyte CD11b and CD62L expression in response to interstitial inflammation in CAPD patients.
Perit Dial Int. 2004; 24 (1):28-36.

IV Dadfar E, Moshfegh A, Paulsson J, Olsson KJ, Jacobson SH, Lundahl J
Gene expression pattern of peripheral and in vivo transmigrated neutrophils in healthy subjects and patients with renal failure.
Manuscript

CONTENTS

LIST OF ABBREVIATIONS

CAPD	Continuous ambulatory peritoneal dialysis
C3b	Complement factor 3b
CD	Cluster of differentiation
DCFH	Dichlorofluorescein diacetate
EDTA	Ethylene diaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ESRD	End stage renal disease
FITC	Fluorescein isothiocyanate
fMLP	N-formyl-methionyl-leucyl-phenylalanine
GFR	Glomerular filtration rate
ICAM	Intercellular cell adhesion molecule
Ig	Immunoglobulin
IL	Interleukin
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MIP	Macrophage inhibitory protein
MHC	Major histocompatibility complex
MCP	Monocyte chemoattractant protein
MFI	Mean fluorescence intensity
MPO	Myeloperoxidase
PBS	Phosphate buffered saline
PE	Phycoerithrin
PECAM	Platelet endothelial cell adhesion molecule
PLAU	Plasminogen activator urokinase
PMA	Phorbol 12-myristate 7-acetate
PMN	Polymorphonuclear cells
PSGL-1	P-selectin glycoprotein ligand-1
THBS1	Thrombospondin 1
TGF	Transforming growth factor
TNF	Tumor necrosis factor
VCAM	Vascular cell adhesion molecules
VLA	Very late activation antigen

1 INTRODUCTION

1.1 NEUTROPHILS

Neutrophils compromise two-thirds of the circulating leukocyte population and constitute the most abundant inflammatory cells in the peripheral circulation. They belong to a group of leukocytes designated granulocytes. Other members of the granulocyte groups are eosinophils and basophils. Neutrophils mature in the bone marrow before being released to the peripheral circulation, where they spend 4-10 hours, before marginating and entering tissue where they can survive for 1-2 days. Senescent neutrophils are known to undergo apoptosis prior to removal by macrophages (Gallin 1999).

The neutrophils constitute the first line of defense against infectious agents that penetrate the body's physical barrier. Upon infection the neutrophils are recruited to the inflamed tissue where they play an important role in host defense against all classes of infectious agents. The major defense mechanisms against bacteria and fungi are phagocytosis, oxidative burst, cytokine production, and release of antimicrobial peptides (Smith 1994). The latter are synthesized during the proliferation step in the bone marrow and stored in distinct cytoplasmic granulae. Antimicrobial peptides include defensins, cathelicidins, protegrins and histatins (Reddy 2004, Van Eeden 1999). IL-8 is a known chemoattractant for neutrophils.

Neutrophils contain four types of granulae: azurophilic granulae (primary), which are the main source for myeloperoxidase (MPO), alpha defensins and proteases, specific granulae (secondary); the main intracellular storage place for lactoferrin, gelatinase, collagenase, CD11b/CD18 and antimicrobial substances; gelatinase granulae (tertiary), which contain the adhesion molecule CD11b/CD18, gelatinase and cytochrome b_{558}. Finally, secretory granulae constitute a reservoir of membrane-associated granulae needed in the earliest phases of the neutrophil mediated inflammatory response. These granulae contain CD11b/CD18, complement receptor 1, N-formyl-methionyl-leucyl-phenylalanine (fMLP) receptors and plasma proteins (Faurschou 2003). A hierarchy in the mobilization of the granulae has been demonstrated, namely secretory granulae, gelatinase granulae, specific granulae and azurophil granulae (Sengelov 1995, Borregaard 1997).

Neutrophils have long been known to be responsible for innate immunity but recently they have also come to be considered intimately associated with the establishment of acquired immunity. Activated neutrophils produce the cytokines CCL3 (Macrophage inhibitory protein 1α, MIP-1α), CCL4 (MIP-1β) and CCL5 (RANTES) that attract immature dendritic cells (DC) and T-cells. Furthermore, alpha

defensins in neutrophil granulae are chemotaxic for immature dendritic cells (Yang 2000). Another important effect of neutrophils to induce acquired immunity (dendritic cell maturation) is by the costimulatory molecules CD40, CD80 and CD86 (Bennouna 2003).

1.2 MONOCYTES

Monocytes constitute 5-10% of peripheral blood leukocytes. Monocytes and macrophages are members of the mononuclear phagocyte system (MPS). They originate from $CD34^+$ myeloid progenitor cells in the bone marrow and are released into the peripheral circulation where they circulate for several days before entering tissues, where they differentiate into macrophages or dendritic cells (Van Furth 1998).

The differentiation of monocytes to macrophages is characterized by substantial changes in adhesion molecule expression and cytokine production (Ammon 2000, Wintergest 1998, Zou 2002, Valledor 1998, Prieto 1994). The local environment significantly influences the function of macrophages, which is mirrored by the fact that cells from different tissues display different patterns of function (Stout R 2004).

Monocytes express CD14 which is a receptor for bacterial lipopolysaccharide (LPS). LPS binding to CD14 initiates transmembrane signalling and changes in cellular function (Ziegler-Heitbrock 1993). Monocytes can be classed into subtypes with heterogeneous phenotypes and functions (Gordon 2005, Imhof 2004 Grage-Griebenow 2001) The main subsets are $CD14^{high}CD16^-$ ”classical” monocytes, which express CCR2 and are recruited to inflammatory foci. $CD14^+CD16^+$ monocytes or “regular monocytes” express higher amounts of MHC class II molecules, CD32 and CCR5. These are resident cell populations that are recruited to the tissues independently of inflammatory stimuli (Ziegler-Heitbrock HWL 1993). An additional monocyte subset is $CD14^+CD16^+CD64^+$ monocytes. These cells combine characteristics of monocytes and dendritic cells with high expression of CD86 and HLA-DR and high T-cell stimulatory activity (Grage-Griebenow 2001).

Peripheral monocytes express three members of the β_2-integrin family, CD11a/CD18, CD11b and CD11c/CD18, as well as the β_1-integrin family (very late antigen-4) VLA-4 and VLA-5 (Meerschaert 1995, Shang 1998, Ehlers 2000, Issekutz 1995). Activated monocytes and macrophages produce the pro-inflammatory cytokines interleukin-1 (IL-1), IL-6, IL-8, IL-12 and tumor necrosis factor alpha (TNF-α), as well as the deactivating cytokines tumor growth factor beta (TGFβ) and IL-10 (Van Furth 1998).

1.3 TRANSMIGRATION AND ADHESION MOLECULES

The migration of leukocytes from the peripheral circulation into infected or injured tissue is a fundamental step in the host-defense mechanism and involves a series of sequential molecular interactions between leukocytes and endothelial cells. The migration can be divided into four events: rolling, activation, tight adhesion and diapedesis (Gallin 1999, Springer 1990), see figure 1.

The adhesion cascade is initiated by selectins (CD62L) that interact with glycoprotein ligands, allowing the leukocyte to bind weakly on the endothelium (Adams 1994) These "rolling" leukocytes are stimulated by chemokines and other activating compounds such as fMLP, platelet activating factor (PAF) and leukotriene B_4. The activation induces firm binding of leukocytes by integrins (CD11b) binding to their counterreceptors (ICAM-1) on endothelial cells (Albelda 1990).

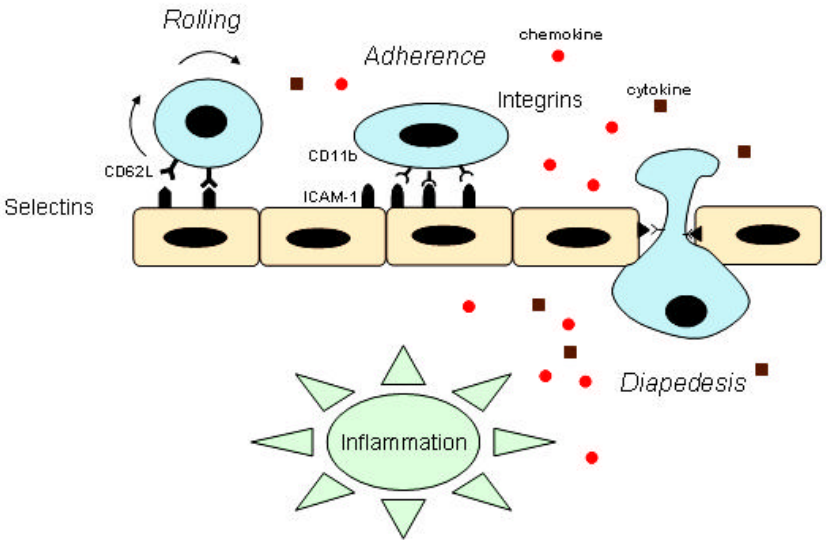

Figure 1 Leukocyte adhesion and transmigration.

The leukocytes then crawl rapidly to an intracellular junction where they transmigrate between the tightly fitting endothelial cells. The transendothelial transmigration is mediated by platelet endothelial cell adhesion molecule-1 (PECAM-1) (Muller W 1999, Imhof 2004).

1.3.1 Selectins

The first interaction between leukocytes and endothelial cells is mediated by selectins. The selectins capture free flowing leukocytes in the blood flow and initiate rolling along the endothelium. Selectins consist of a family of three: L-selectin, P-selectin and E-selectin that share three common extracellular domains; a calcium-dependent lectin domain, an epidermal growth factor domain and a short consensus repeat domain. The ligand binding ability of selectins is predominantly mediated by the

lectin domain. The sialyl Lewis binds to each of the selectins and has therefore been identified as a prototype selectin ligand (Robinson L A 1999, Patel 2002).

L-selectin (CD62L, LAM-1) was first identified as a lymphocyte homing receptor (Gallatin 1983). It is expressed on all classes of leukocytes at most stages of differentiation. Although it is rapidly expressed on the leukocytes it is also rapidly shed, by membrane bound protease. Five L-selectin ligands have been identified. They are GlyCAM-1, MadCam-1, CD34 and Sgp200, and PSG-1 (Vestweber 1999, Rosen 1994).

P-selectin (CD62P, LECAM-3) is stored in the α-granules of platelet and in the Weibel Palade bodies of endothelial cells. Activating agents such as histamine, lipopolysaccharide (LPS), TNF-α, IL-4, and IL-13 induce translocation of P-selectin to the cell surface. P-selectin is short lived and mediates early leukocyte endothelial interactions (Ley 1994). The ligand for P-selectins is P-selectin glycoprotein ligand-1 (PSGL-1).

E-selectin (CD62E, ELAM-1) is expressed on activated endothelial cells. The E-selectin is transcriptionally regulated by mediators such as IL-1 and TNF-α. Peak expression of E-selectin occurs within 4 hours after activation and declines within 24 hours by internalization and degradation of receptors. E-selectin may mediate adhesion in a later phase of inflammation. The ligands for E-selectins are PSGL-1 and E-selectin ligand 1 (Robinson 1999).

1.3.2 Integrins

The term integrins was originally coined to describe membrane receptors that integrate the extracellular environment (matrix or other cells) with the intracellular cytoskeleton. All integrins are αβ heterodimers, consisting of one α and one β domain, non-covalently associated with each other. The integrins are divided into three subfamilies based on their β subunits that share multiple α-subunits (Larson 1990).

The β_1-integrin is expressed in majority of mammalian cells, except in mature erythrocytes. The β_1-family includes many extracellular matrix receptors for fibronectin, laminin and collagen (Rouslahti 1991). The most abundant integrin found on leukocytes is β_2 (CD18). At least three different α subunits have been known to noncovalently bind to β_2 integrin. They include $\alpha_L\beta_2$ (CD11a/CD18), $\alpha_M\beta_2$ (CD11b/CD18), and $\alpha_X\beta_2$ (CD11c/CD18). CD11a/CD18 is predominantly expressed on lymphocytes, while monocytes, macrophages and neutrophils express CD11b/CD18 and CD11c/CD18.

In unstimulated granulocytes and monocytes CD11b/CD18 is stored in intracellular granulae. The CD11b/CD18 shows a marked increase after leukocyte activation by agonists such as phorbol esters, fMLP, granulocyte macrophage colony stimulating factor (GM-CSF), TNF-α, C5a and LTB_4 (Carlos 1984). An elevated expression of CD11b/CD18 has also been noted in neutrophil adhesion to E-selectin (Lo 1991). The CD11b/CD18 is a sensitive marker of cell activation and initiates the firm adhesion of transmigration.

In addition to the transmigration process, the CD11b/CD18 plays an important part in C3b opsonization, H_2O_2 secretion, regulation of phagocytosis and apoptosis of leukocytes (Kishimoto 1999). The importance of CD11b is demonstrated in patients with leukocyte adhesion deficiency type I (LAD I). These patients have a defect synthesis of the β_2 integrin and they acquire recurrent life-threatening bacterial and fungal infections and have poor wound healing. The cellular defect in LAD is manifested as impaired leukocyte adherence, migration, and phagocytosis (Ehlers MR 2000, Hogg 1999). Ligands for β2 integrins include ICAM-1, ICAM-2 and ICAM-3 (Dustin 1998) as well as soluble proteins such as fibrinogen, factor X and complement fragment (Arnaout N 1990). Neutrophil and monocyte transmigration relies on CD11a-c/CD18 while lymphocytes express primarily CD11a/CD18 (Carlos 1984). In this thesis we have focused on CD11b/CD18 expression on peripheral and in vivo transmigrated neutrophils and monocytes.

1.3.3 Immunoglobulins

The immunoglobulin superfamily is expressed on endothelial cells and serves as counter receptors to integrins. The immunoglobulins ICAM-1 and ICAM-2 are recognized by β2 integrins whereas vascular cell adhesion molecules (VCAM-1) are recognized by β1 integrins. Immunoglobulins have also been shown to participate in transendothelial migration by platelet endothelial cell adhesion molecule-1 (PECAM-1) (Muller 1993).

1.4 RESPIRATORY BURST

Human phagocytes eliminate microorganisms by phagocytosing pathogens and exposing them to reactive oxygen species. Oxygen is utilized by the powerful enzyme NADPH oxidase to produce superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2). The activity of the enzyme must be carefully regulated, in order to prevent tissue injury. Therefore, the NAPH oxidase consists of one membrane-bound and four cytosolic subunits, located in different parts of the neutrophils. Upon cellular activation, the cytosolic subunit translocates to the membrane-bound component and assembles into the active enzyme NADPH oxidase. In the phagosome, oxygen (O_2) is reduced to superoxide (O_2^-) by

NADPH oxidase. The superoxide is reduced to hydrogen peroxide (H_2O_2) in the presence of protons (Roos 2003, Dahlgren 1999). Myeloperoxidase catalyzes the reaction from H_2O_2 to other potent oxidizing radicals (fig 2). Activation of respiratory burst in leukocytes has been shown to correlate with adhesion dependent signaling and stimulation such as TNFα, PAF and fMLP (Dapino 1993, Yan 1999)

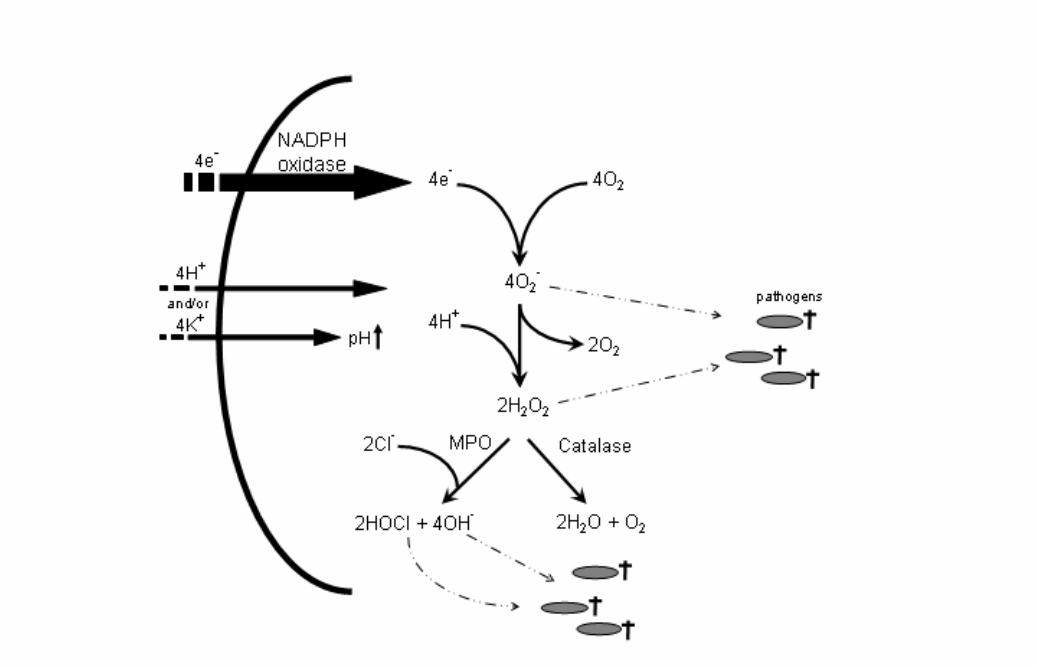

Figure 2 Oxidative burst

1.5 CHEMOKINES AND CYTOKINES IN INFLAMMATION

Cytokines are redundant secreted proteins involved in cell growth, differentiation and activation. Cytokines exert their actions in an autocrine, paracrine or endocrine manner. The leukocyte migrates from the peripheral circulation into the tissues in response to a gradient of chemoattractants. Chemoattractants include bacterial products (e.g. fMLP), products from the complement cascade (C3a, C5a), secreted products of stimulated phospholipid metabolism (e.g. PAF, LTB_4) and chemokines. Chemokines are small (5-20 kDa) proteins that are released by a vast number of cells. They can be considered as proinflammatory cytokines with chemotactic properties. They are characterized by the conserved position of four cysteine residues. Based on the relative position of the cysteine residues the chemokines can be divided in two subfamilies, CXC and CC chemokines. Most CXC chemokines have strong neutrophil chemotactic and activating properties while many CC chemokines specifically attract macrophages and T-cells.

The cytokines and chemokines analyzed in this thesis are presented in table 1.

1.6 PATIENTS WITH RENAL FAILURE

The major causes of chronic kidney disease (CKD) are glomerulonephritis, diabetes mellitus, nephrosclerosis, interstitial nephritis and polycystic kidney disease. Renal function can be measured indirectly by determination of the concentration of creatinine in serum or directly by measurement of glomerular filtration rate (GFR). Measurement of creatinine concentration in serum is the most commonly used screening test for renal function. It is fast and simple; however, as much as 50% of the nephrons may be lost before the creatinine levels increase. In addition the levels of creatinine are influenced by extra-renal elimination, muscular mass, body mass, age and diet. Glomerular filtration rate (GFR) can be estimated indirectly by using various formulas. The most widely used and accepted methods to predict GFR in adults are the proposed by Cockcroft and Gault (Cockcroft 1976) and the Modification of Diet in Renal Disease (MDRD) (Levey 1999). New methods for estimating GFR have been introduced, e.g. a GFR prediction based solely on cystatin C (Grubb 2005, Rodrigo 2002). The National Kidney Foundation in the United States classes chronic kidney disease in five stages based on level of GFR. Stage 1 represent normal or elevated GFR and stage 5 represents a GFR less than 15 mL/min or treatment with dialysis. The different stages are presented in table 2.

Stage	Description	GFR ml/min/1.73m^2
1	Kidney damage with normal or increased GFR	>90
2	Kidney damage with *mildly* reduced GFR	60-89
3	*Moderately* reduced GFR	30-59
4	*Severely* reduced GFR	15-29
5	Kidney failure	<15 or dialysis

Table 2 Classification of chronic renal disease according to National Kidney Foundation.

When renal function declines many patients develop hypertension, renal anemia, secondary hyperparathyroidism and subjective symptoms related to the retention of a number of uremic toxins. Uremia is clinically manifested by anorexia, malaise, vomiting, headache, anemia, malnutrition and endocrine disturbances (Kasper 2003). When end-stage renal disease (ESRD) has developed (GFR <15 mL/min), but prior to the start of renal replacement therapy (hemodialysis, peritoneal dialysis or kidney transplantation), many patients show signs of malnutrition. From this stage and during dialysis

patients are at increased risk of developing cardiovascular disease and infections: together this is called the MIA syndrome (**m**alnutrion, **i**nflammation and **a**therosclerosis) (Stenvinkel 2000).

1.6.1 Renal failure and increased susceptibility to infections

Patients with chronic renal failure are highly susceptible to infections. Impairment of the host defense is primarily responsible for the increased susceptibility and one contributing factor is dysfunctional polymorphonuclear cells (Cohen 1997, Hörl 1990). The dysfunctionality is manifested as reduced chemotaxis, decreased phagocytic ability, reduced intracellular killing (Muniz-Junqeira 2005, Lewis 1987*)* and increased apoptotic rate (Jaber 2001, Heidenreich 1996, Jaber 2001).

In addition, these patients display extended survival of skin allografts (Sester 1997), reduced immunization against hepatitis B (Köhler 1984), influenza (Rautenberg 1989), diphtheria (Kreft B 1997) and tetanus vaccine (Girndt 1995), which indicates an immune defect in antigen presenting cells (Girndt 2001). Malnutrition, loss of vitamins, iron overload, secondary hyperparathyroidism, and uremic toxins also contribute to leukocyte dysfunction (Hörl 1999, Hörl 1990).

1.7 SKIN CHAMBER TECHNIQUE

The migration of polymorphonuclear leukocytes into tissues is a fundamental step in the host defense mechanism. The transmigration and inflammatory process is extremely complex and includes several underlying processes that are linked to each other. Most studies of leukocyte recruitment are done using various in vitro techniques, which have limited clinical relevance. In contrast, the skin chamber technique studies the innate immunity and the transmigration of leukocytes in vivo (Follin 1999, Theiligaard-Mönch 2004, Hellum 1977, Fiuza 2000). The skin chamber technique is a well documented method that provides a means to study local leukocyte exudation without systemic inflammatory responses. A local inflammatory reaction induces the leukocytes to leave the blood stream and migrate to sites where they can be collected.

The skin blisters are formed by removing the epidermis from the underlying dermis with a low negative pressure (Kiistala and Mustakallio 1964). These blisters are produced without damaging capillaries or tissues. The roof of the blisters is removed and a skin chamber containing chemoattractants is applied over each skin lesion (fig 3). The accumulation and activity of leukocytes are highly dependent on the chemoattractant used and the composition of the fluid in the chamber. Attractants used are serum (Kuhns 1992), plasma (Elmegren 1985) and heat inactivated E-coli (Fiuza 2000). Application of skin chamber technique and subsequent analysis of temporal changes in the cell

population in the chamber reveal that mononuclear cells appear early but are soon outnumbered by polymorphonuclear leukocytes, which constitute 90-98% of the cells after 10-24 h (Kuhns 1992).

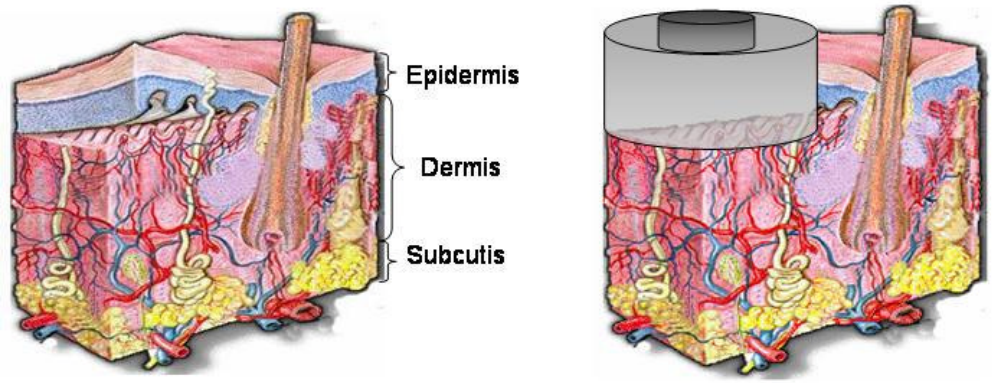

Figure 3 The position of the skin chamber (*modified from Mosbys Atlas*).

2 AIMS OF THE STUDY

The objective of this thesis was to analyze the transmigration and state of activity of neutrophils and monocytes in healthy subjects and patients with renal failure, using the skin chamber technique. The specific aims were

I. To determine the state of activity of in vivo transmigrated monocytes in healthy subjects, prior to macrophage differentiation, in terms of CD11b upregulation and intracellular hydrogen peroxide production.

II. To study the recruitment of monocytes and their expression of adhesion molecules CD11b and CD62L at the site of interstitial inflammation in patients with renal failure, and to investigate whether the capacity of monocytes to up regulate CD11b is determined by the chemotactic factors in the interstitial milieu.

III. To study the recruitment of monocyte and granulocytes to inflammatory foci in patients on peritoneal dialysis, using the skin chamber technique, and to examine the cells' ability to modulate the expression of adhesion molecules CD11b and CD62L.

IV. To study the gene expression pattern of peripheral and in vivo transmigrated neutrophils in patients with renal failure and healthy subjects, with special attention focused on genes involved in chemotaxis.

3 MATERIAL AND METHODS

3.1 SUBJECT CHARACTERIZATION

Patients with renal failure were recruited from the Department of Nephrology at Karolinska University Hospital. All participants gave their informed consent and the study was approved by the Ethics committee of the Karolinska Hospital. All patients and healthy subjects suffering from infectious diseases, diabetes mellitus or active inflammatory diseases as well as those receiving antibiotics, corticosteroids or non-steroidal anti-inflammatory agents were excluded from the studies.

Study I

12 healthy subjects (7 women, 5 men) with a median age of 53 (interquartile 49-57) years participated in this study. Samples from peripheral blood and skin blister exudates were collected from these subjects.

Study II

Ten patients (7 males, 3 females) with a median age of 59 (50-72) years with impaired renal function participated in this study. Patients had a serum creatinine of 453 (236-694) µmol/L and an estimated GFR level of 11.7 (7.8-25.1) mL/min (according to the Cockcroft and Gault equation). The renal diagnoses were the following: 4 patients had glomerulonephritis, 4 had nephrosclerosis and 2 had polycystic kidney disease.

Study III

Ten patients on peritoneal dialysis (7 men and 3 women, median age 56 years, range 48-70 years) participated in this study. The patients had been on peritoneal dialysis for a median time of 11 months (7-23 months).

Controls (II-III)

Nineteen healthy subjects with a median age of 32 (28-40) years with an estimated GFR level of 94.7 (89.4-95.1) mL/min participated simultaneously in study I and II.

Study IV

The study population consisted of six patients with severe renal failure (GFR <20 mL/min according to the Cockroft and Gault equation) and six healthy subjects. The healthy subjects were age and sex matched with the patients. The renal diagnoses were the following: three patients had nephrosclerosis, one had polycystic kidney disease and two had renal failure of unknown origin.

3.2 IN VIVO METHODS

3.2.1 Skin chamber technique

In paper I-III two 9 mm skin blisters were raised on the volar surface of the forearm by gentle suction (300 mm Hg) (Thylén 2000). The blisters were covered overnight with a plastic eye chamber (Augenverband S; Lohmann GmBh, Munich, Germany). The next morning the blister fluids were aspirated and pooled. The pool of blisters was designated “time 0 h” and represents the unstimulated skin blisters. The roofs of the blisters were then gently removed and a transparent sterile adhesive plastic film with a 10 mm diameter hole was applied around the exposed blister floors (Tegaderm; 3M Pharmaceuticals, Loughborough, England). A sterilized open-bottom plastic skin chamber with an inner volume of 1 mL was placed over each skin lesion and secured. In order to induce different intensities of inflammation, the blisters were stimulated with PBS (intermediate inflammation) or heparinized autologous serum (intense inflammation). Autologous serum was chosen as chemoattractant as it contains biologically active components from the coagulation and complements systems and has been shown to be a potent leukocyte mobilization factor (Follin 1999). The autologous serum was collected the day before, centrifuged for 15 min at 4°C and immediately frozen at -70°C. After 10 hours of incubation the fluid was aspirated from each chamber and placed on ice. In paper I the chambers were washed with 1 mL PBS in order to increase the number of collected cells.

In paper IV three skin chambers were introduced. Briefly, two skin blisters were raised on the volar surface of the forearm as described above. After formation of the two blisters a third skin blister was introduced at the lower surface of the forearm with the same procedure. The blisters were covered overnight with a plastic eye chamber. The following morning the roof of each blister was carefully removed and an open bottom plastic skin chamber (volume 1 mL), was placed over each unroofed blister. One milliliter of heparinized autologous serum, collected the day before, was added to each chamber. After 10 hours of incubation the interstitial exudates were aspirated from each chamber and placed on ice. The chambers were washed with 1 mL PBS which was then pooled with the other fluid from the respective chambers.

The blister exudates [I-IV] were centrifuged at 300 × g for 5 min at 4°C, the supernatants were aliquoted in 200 µl portions and immediately frozen at -70°C. The pellets were resuspended in 500 µl PBS, pH 7.4 and kept on ice until used. The rationale for choosing 10 hours of incubation was based on previous studies (Thylén 2001, Jacobson 2002) in which we observed that a sufficient number of cells transmigrate during 10 hours.

3.3 IN VITRO METHODS

3.3.1 Preparation of leukocytes

[I, II, III]

Blood samples were collected in the morning when the first pool of blister exudate was collected (see above) and 10 hours thereafter in glass tubes containing EDTA (Vacutainer, 5 mL, with 50 μL of 21% EDTA, Terumo) [II, III] or citrate (Vacutainer, 5 mL, with 1 mL 0.129 M 9NC, Becton Dickinson) [I, IV]. The blood samples were divided in 100 μL portions and erythrocytes were hemolyzed by addition of 2 mL 4°C isotonic NH_4Cl-EDTA "lyzing solution" (containing 154 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.2). After 5 min incubation at 4ºC the cells were centrifuged at 300 g for 5 min at +4ºC. The leukocyte suspension was washed with 2 mL +4°C PBS supplemented with 2 mL +4°C 0.02% NaN_3 (PBS-azide, paper I) or with PBS supplemented with 0.1 mM EDTA and 0.02% NaN_3 (PBS-EDTA, paper II and III) before immunostaining.

[IV]

Peripheral neutrophils were purified from 20 mL blood by Ficoll separation followed by immunomagnetic depletion of non granulocytic cells. Briefly, 20 mL blood was diluted once with PBS, layered on 25 mL Percoll (Pharmacia & Upjohn, Uppsala, Sweden) and centrifuged at 400 g for 20 min at room temperature. After centrifugation the mononuclear cell layer was separated from the polymorphonuclear cells (PMNs) and red blood cells. The red blood cells were hemolyzed by addition of 40 mL lyzing solution. The PMNs were pelleted and washed with PBS (300 × g, 4ºC, 10 min). A positive selection of neutrophils was performed by incubating the PMN with anti-CD16 coupled to MACS beads (Miltenyi Biotec, Auburn, California, USA). The cells were then loaded onto a MidiMACS column and the CD16 positive neutrophils were collected. The purity of the neutrophil fraction was analyzed by flow cytometry (forward and side scatter properties), and only fractions above 97% purity were taken into consideration.

3.3.2 Determination of soluble mediators

The levels of MCP-1 [I-III], IL-8 [II, III], IL-6 [I], and TNF-α [I] in serum and blister exudates were measured with commercially available immunoassays (Quantikine human immunoassay; R&D Systems, Minneapolis, Minnesota, USA). The assays were performed according to the manufacturer's instructions.

3.3.3 Respiratory burst assay [I]

When dichlorofluorescein diacetate (DCFH-DA) (Eastman Kodak Company, Rochester, NY) permeates the leukocyte membrane it is oxidized by hydrogen peroxide to fluorescent 2'7'-dichlorofluorescein. The amount of hydrogen peroxide produced can be quantified by measuring

the fluorescence intensity by flow cytometry. To get a proper permeation of cell membrane, leukocytes were incubated with PBS-glucose supplemented with 5 μM (5 μmol/L) DCFH-DA for 15 min at 37°C. During this time the tubes were stirred several times.

In order to study the H_2O_2 production during cellular activation the samples were stimulated with PBS glucose for 15 min at 4ºC, 2.5×10^{-7}M fMLP (Sigma Chemical, St. Louis, Missouri, USA) for 30 min at 37°C or 2.5×10^{-7} M phorbol-12-myristate 7-acetate (PMA) (Sigma Chemical) suspended in PBS-glucose for 15 min at 37ºC. Cells incubated in PBS glucose served as controls. Activation was terminated by addition of 1 mL ice cold PBS-EDTA. Cells were subjected to flow cytometry to determine H_2O_2 production.

3.3.4 In vitro activation of leukocytes [I]

In order to study the ability to mobilize the adhesion molecule CD11b, leukocytes from serum and blisters were incubated for 15 min at 4°C in RPMI with 5% fetal calf serum (PAA Laboratories, GmbH, Austria) or with 5×10^{-7} M fMLP (Sigma, St. Louis, Missouri, USA) for 15 min at 37°C. After incubation, the cell suspensions were washed once in 2 mL PBS-azide (300 × g for 5 min, 4ºC) and resuspended in 100 μL PBS-azide before immunostaining.

3.3.5 Immunostaining [I-III]

The CD11b expression on monocytes and granulocytes was analyzed by adding 5 μL of phycoerythrin conjugated monoclonal anti-CD11b (final concentration 5μg/mL) (Dako AS, Glostrup, Denmark). The expressed CD62L was immunostained by addition of 10 μL of FITC conjugated anti-Leu 8 (Becton & Dickinson, Immunocytometry Systems, CA, USA) to the leukocyte pellet in 100 μL PBS. Appropriate concentrations of an isotype matched control antibody were used to define the cut-off for positive fluorescence, which was the 99th percentile of the distribution of the cells labeled with the respective control antibody (PE conjugated $IgG2_a$ (Dako) and FITC conjugated $IgG2_a$ (Becton & Dickinson) for CD11b and CD62L respectively). The cells were incubated for 30 min, washed once in 3 mL cold PBS-EDTA [II-III] or PBS [I] and finally resuspended in 0.5 mL cold PBS EDTA [II-III] or PBS [I] before analysis by flow cytometry.

3.3.6 Leukocyte Count

To determine the number of leukocytes in the peripheral circulation, 100 μL blood was hemolyzed, stabilized and fixed according to the Multi-Q-prep ImmunoPrep technique (Beckman Coulter, Inc. Hialeah, Florida, USA). The number of cells was then counted by flow cytometry. In the blister exudates, leukocytes were counted directly using flow cytometry.

3.3.7 Analysis of leukocytes by flow cytometry [I-IV]

The cells were analyzed by Epics XL flow cytometry (Beckman Coulter Inc., Hialeah, Florida, USA). This instrument gives the actual number of cells and the mean fluorescence intensity (MFI), which represents the density of the antigens of the cell population within a chosen field. The granulocyte and monocyte cell populations were distinguished by their different light scattering properties (Forward Scatter (FS)/Side Scatter (SS)). The forward scatter signal (y-axis) reflects the cell size while the side scatter signal (x-axis) reflects cell granularity. The granulocytes and monocytes were gated in separate clusters, and a minimum of 300 cells were counted during analysis. The percentage and absolute number of positively immunostained leukocytes was determined by measuring MFI of the positive cell population.

The instrument was calibrated each day with 10 mm standardized fluorospheres, Flow Check (Beckman Coulter). Another fluorosphere (Flow Set, Beckman Coulter), with controlled fluorescence intensity, was used before each experiment to obtain a standardization of the MFI.

3.3.8 Gene array [IV]

Sample preparation and processing procedure was performed at the Microarray Core Facility, Cancer Centre Karolinska at Karolinska University Hospital. The preparation was performed according to Affymetrix GeneChip Expression Analysis Manual (Affymetrix Inc., Santa Clara, UK). Briefly, total RNA from purified neutrophils was extracted according to Qiagen RNAeasy kit (WVR, Stockholm, Sweden). The total RNA quality was confirmed by Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA). Two cycles, double-stranded cDNA was synthesized from 50 ηg total RNA with SuperScript Choice system (Invitrogen Inc.). The cDNA pellet was collected and dissolved in appropriate volume according to the Affymetrix protocol. Using cDNA as template, cRNA was synthesized by In-Vitro Transcription (IVT) kit (Affymetrix Inc). Biotinylated CTP and UTP ribonucleotides (Enzo Diagnostics Inc., Farmingdale, New York, USA) were added to the reaction as labeling reagents. The IVT reactions were carried out at 37°C for 5 hours and labeled cRNA was obtained.

The cRNA was fragmented by a fragmentation buffer (40 mmol/L Tris-acetate, pH 8.1, 100 mmol/L KOAc, 30 mmol/L MgOAc) for 35 minutes at 94°C. The fragmented cRNA (15 μg/probe array) was used to hybridize human U133A GeneChip array at 45°C for 18 hours in a hybridization oven with constant rotation (60 rpm). After hybridization, chips were washed and stained using Affymetrix fluidics stations. Staining was performed using streptavidin phycoerythrin conjugate (SAPE; Molecular Probes, Eugene, USA). This was followed by addition of biotinylated antibody to streptavidin (Vector Laboratories, Burlingame, USA) and finally with streptavidin phycoerythrin

conjugate. Probe arrays were scanned using fluorometric scanners (Agilent Gene Array Scanner; Agilent Technologies). The scanned images were inspected and analyzed using established quality control measures.

3.3.9 Real-Time PCR [IV]

RNA from purified neutrophils was extracted according to Qiagen RNAeasy kit (WVR). The integrity of total RNA was confirmed by Agilent 2100 Bioanalyzer (Agilent Technologies). Using 50 ng of total RNA, single-stranded cDNA was synthesized following SuperScript Choice system (Invitrogen Inc.).

Gene expression was measured with the use of ABI 7500 thermocycler (PE Applied Biosystems). Primers and probes for CCL2, CCL3, CCL4, IL-8 and the housekeeping gene GAPDH were purchased from Applied Biosystems. Probes were labeled at the 5'end with the reporter dye molecule FAM (6-carboxy-fluorescein) and at the 3'end with quencher dye molecule TAMARA (6-carboxytetramethylrhodamine). Target genes and housekeeping gene (GAPDH) were simultaneously tested in duplicates.
Real-time PCR of cDNA specimens was conducted in a total volume of 25 µl with 2× TaqMan Master Mix (Applied Biosystems, Warrington, UK), primers at 300 nM and probes at 200 nM.

The relative quantitative expression of the genes was determined by using the arithmetic equation $2^{-\Delta\Delta CT}$ according to Applied Biosystems (1997). The amount of RNA was normalized to the endogenous reference gene (GAPDH) at each stage in order to distinguish differences in the total amount of nucleic acid added to a reaction mixture. The values are expressed relative to a reference sample.

3.4 STATISTICAL ANALYSIS

3.4.1 Paper [I-III]

Results are expressed as median and interquartile ranges. Statistical comparisons were made using Wilcoxon matched pairs test and Mann–Whitney U-test. The correlation analysis was done using Spearman Rank test.

3.4.2 Paper [IV]

3.4.2.1 Gene array data analysis

The expression analysis file created from each sample (chip) scanning was imported into GeneSpring 7.2 software (Agilent, Redwood City, USA) for further data characterization. The data

were normalized by using the 50th percentile of each chip (per chip normalization). Intensity range, expression values and relative expression data for each set of probes were generated by normalization over the median of the entire experiment set (per gene normalization). Data filtration was generated based on flags present in at least one of the samples. Lists of genes included genes that were either induced or suppressed >1.5-fold between patient and healthy control and/or blood and blister. The gene lists were categorized according to their biological functions as described in GeneSpring, NetAffx database (Affymetrix) and GeneCards.

4 RESULTS AND COMMENTS

4.1 IN VIVO TRANSMIGRATED MONOCYTES STATE OF ACTIVITY, IN HEALTHY SUBJECTS (I)

4.1.1 Number and cellular constitute of in vivo transmigrated leukocytes

The migration of leukocytes from the peripheral circulation into infected or injured tissue is a fundamental step in host defense. In this study, we evaluated the number of leukocytes that transmigrated in response to different intensities of induced inflammation in the skin in vivo by use of the skin chamber technique. In order to induce different degrees of inflammation, the skin blisters were stimulated with PBS (representing intermediate inflammation) or autologous serum (representing intense inflammation). The number of transmigrated leukocytes to the unstimulated blister (0 hours) was 0.3×10^6 ($0.2\text{-}0.5\times10^6$). The number increased significantly in the skin chamber stimulated with PBS (1.2×10^6 ($0.7\text{-}1.4\times10^6$)) and serum (3.6×10^6 ($2.4\text{-}4.7\times10^6$)). The highest number of transmigrated cells was observed at the site of intense inflammation. There was no correlation between the number of cells in the peripheral circulation and the number of transmigrated leukocytes. The cellular composition in blisters differed from that in the peripheral circulation (table 4). The blister exudates were dominated by neutrophils followed by monocytes and lymphocytes. Similar cellular distributions have been observed by other groups (Scheja A 1985, Kuhns 1992, Follin 1999).

	Peripheral circulation	Unstimulated blister Time 0 h	p-value	Blister stimulated with PBS	p-value	Blister stimulated with serum	p-value
Total cells	6.7×10^9 ($5.1\text{-}7.9\times10^9$/L)	0.3×10^6 ($0.15\text{-}0.4\times10^6$/L)		1.2×10^6 ($0.7\text{-}1.4\times10^6$/L)		3.6×10^6 ($2.4\text{-}4.7\times10^6$/L)	
% Granulocytes	60.1 (53.2-64.0)	75.1 (69.8-77.6)	NS	85.8 (76.5-88.6)	p<0.005	85.4 (80.9-86.6)	p<0.01
% Monocytes	8.5 (6.7-9.2)	19.3 (17.9-24.3)	p<0.01	11.1 (8.1-17.7)	p<0.05	11.6 (8.1-12.3)	NS
% Lymphocytes	31.8 (26.8-36.3)	6.0 (4.9-8.4)	p<0.05	3.5 (2.4-5.5)	p<0.005	2.1 (1.2-6.6)	p<0.05

Table 4. The cellular distributions in peripheral circulation and in skin blister exudates in healthy subjects. The data are presented as median ± interquartile range. The p value reflects the statistical differences of the site of inflammation and peripheral circulation.

4.1.2 CD11b/CD18 expression during transmigration

Transmigration induced a rapid and pronounced increase in CD11b expression on leukocytes. The baseline CD11b expression (4ºC) was five fold higher in transmigrated monocytes in the skin chamber compared to in monocytes collected from the peripheral circulation ($p<0.05$). In order to mimic the monocyte response to a bacterial peptide, monocytes were stimulated with fMLP. In vitro

activation of peripheral and transmigrated monocytes with fMLP induced an increase in the CD11b expression ($p<0.01$). The fMLP induced CD11b expression was higher in the skin chambers than in the peripheral circulation (fig 5). This indicates that monocytes at the interstitial site of inflammation increase their responsiveness towards fMLP.

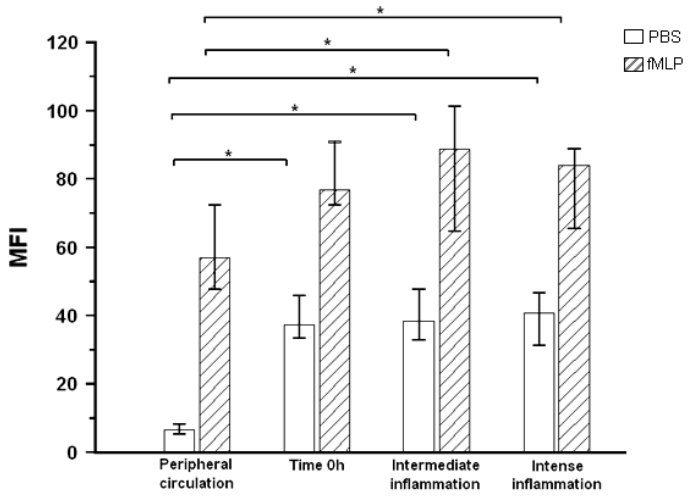

Figure 5. CD11b expression, at 4ºC and with fMLP stimulation, in peripheral and in vivo transmigrated monocytes from healthy subjects. The results re presented as median and interquartile range.*=p<0.05

4.1.3 Respiratory burst in peripheral and transmigrated monocytes

In order to study the intracellular killing capacity, the respiratory burst in monocytes at 4ºC and after in vitro activation with fMLP and PMA was measured. Incubation with fMLP and PMA induced a significant increase in oxidative burst. Transmigrated monocytes had similar H_2O_2 production following fMLP stimulation but a reduced H_2O_2 response to PMA, as compared with peripheral monocytes. Thus, in vivo transmigrated monocytes seem to preserve their capacity to respond to fMLP in terms of CD11b upregulation and H_2O_2 generation.

4.1.4 Proinflammatory cytokines in serum and blister exudates and their relation to CD11b expression

The impact of the interstitial environment on CD11b expression on monocytes was studied by measuring the concentrations of the proinflammatory cytokines TNF-α, IL-6 and MCP-1 in blister exudates, and their correlation with the cellular CD11b expression. All cytokines showed an increased concentration (10-1000 fold) at the interstitial site compared to serum (table 5). The highest concentration of cytokines was observed at the site of intense inflammation, which is in line with data from other groups (Zweiman B 1997, Kuhns DB). A positive correlation was found between the

concentration of TNF-α and the CD11b expression on monocytes at the interstitial site. One plausible explanation for this observation is that CD11b integrins on monocytes transmit secondary signals that markedly enhance expression of TNF-α (Fan 1993).

Cytokine	Peripheral circulation	Intermediate inflammation	Intense inflammation
IL-6	1.8 (0.98-3.5)	7821 * (3458-14132)	9482 * (6041-14421)
MCP-1	305.7 (239.7-409.4)	3083.4 * (2049.0-4626.8)	8292.4 *‡ (5534.9-9975.2)
TNF-α	0.7 (0.6-1.2)	152.8 * (34.3-209.9)	396.3 * (308.9-768.0)

Table 5. Cytokine expression in peripheral circulation and in blister exudates. The results are presented as median and interquartile range, *p<0.05 vs peripheral circulation. ‡ p<0.05 vs intermediate inflammation.

4.2 TRANSMIGRATION AND EXPRESSION OF ADHESION MOLECULES ON MONOCYTES IN PATIENTS WITH SEVERE RENAL FAILURE (II)

4.2.1 Number of transmigrated monocytes

Patients with renal failure have an increased susceptibility to infections and this is thought in part to be caused by dysfunctional monocytes. We therefore investigated the number of in vivo transmigrated monocytes in patients with severe renal failure (GFR 12 (8-25) mL/min) not undergoing dialysis. The number of monocytes that transmigrated into the three different blisters were similar in patients with renal failure and healthy subjects (NS).

4.2.2 CD11b and CD62L expression in peripheral and in vivo transmigrated monocytes

Peripheral monocytes from patients with renal failure had a lower CD11b expression compared to corresponding cells from healthy subjects. Upon transmigration, the CD11b expression on monocytes increased significantly both in healthy subjects and patients. However, monocytes from patients with renal failure were unable to upregulate CD11b to same extent as transmigrated monocytes from healthy subjects. The difference was statistically significant at the sites of intermediate ($p<0.005$) and intense inflammation ($p<0.001$).

The CD62L belongs to the selectin family and plays an important role in initiating rolling in the transmigration process. In the peripheral circulation, CD62L expression on monocytes from patients was lower than in corresponding cells from healthy subjects. Upon transmigration the CD62L is shed

from the cell surface. Transmigrated monocytes had a significantly reduced expression of CD62L compared to monocytes in the peripheral circulation. As opposed to the situation in the peripheral circulation, the CD62L expression on monocytes at the sites of intermediate and intense inflammation was significantly higher in patients compared with that in healthy subjects, indicating that monocytes from patients are unable to shed CD62L to same extent as cells from healthy subjects.

The CD11b/CD62L ratio represents the capacity of monocytes to switch into a $CD11b^{high}/CD62L^{low}$ phenotype in response to transmigration and inflammation. This ratio was significantly lower on monocytes from patients at the site of intermediate and intense site of inflammation compared to monocytes from healthy subjects.

4.2.3 MCP-1 concentration in serum and blister exudates

MCP-1 is a potent monocyte activator, which induces upregulation of CD11b (Nicolson G 2005, Roberts PJ). In order to study whether the reduced CD11b expression in patients is due to a low MCP-1 concentration, MCP-1 was determined in skin blister exudates. In patients with renal failure, the concentration of MCP-1 was significantly lower in the blister stimulated with buffer than in corresponding buffer-stimulated blisters from healthy controls ($p<0.05$). In the blisters stimulated with serum the concentration of MCP-1 was also lower in patients but the difference did not reach statistical significance ($p=0.062$).

Inhibition of MCP-1 activity in blister exudates, with a monoclonal antibody, showed no changes in the CD11b expression in patients or healthy subjects, indicating that the lower MCP-1 concentration in blister exudates may not be a major factor in the reduced CD11b expression observed in patients with renal failure.

4.2.4 The biological effect of blister exudates on CD11b expression

In order to determine the local biological activity of blisters in terms of CD11b mobilizing factors, monocytes from healthy subjects were incubated with pooled exudates from the intermediate and intense skin blisters exudates respectively. Blister exudates from patients with advanced renal failure had the same ability to upregulate CD11b as blister exudates from healthy subjects. This indicates that the reduced CD11b expression observed at the interstitial site is not mainly caused by the interstitial milieu. Cellular factors may play a role.

4.3 TRANSMIGRATION AND EXPRESSION OF CD11B AND CD62L ON LEUKOCYTES FROM CAPD PATIENTS (III)

4.3.1 Number of in vivo transmigrated leukocytes

The number of monocytes in the peripheral circulation was significantly higher in CAPD patients compared to healthy subjects ($p<0.01$). An increased number of monocytes were also observed in the unstimulated skin blister at time 0 h. There were no differences in the number of transmigrated monocytes between patients and healthy subjects at sites of intermediate and intense inflammation. There were no differences between patients and healthy subjects in the number of granulocytes in the peripheral circulation or granulocytes that transmigrated into the three different skin blisters. In conclusion, the capacity of leukocytes to migrate into sites of inflammation in the interstitium does not seem to be disturbed in patients on CAPD.

4.3.2 CD11b expression in granulocytes and monocytes from the interstitium

Peripheral granulocytes from patients with renal failure had the same ability to upregulate CD11b as cells from healthy subjects. When the skin blisters were stimulated with buffer or serum, the expression of CD11b on granulocytes increased significantly, both in healthy subjects and CAPD patients. However, granulocytes collected from patients were unable to upregulate the CD11b expression to the same extent as cells from healthy subjects at the site of intermediate and intense inflammation (fig 6). Thus, granulocytes from CAPD patients seem to require a more intense

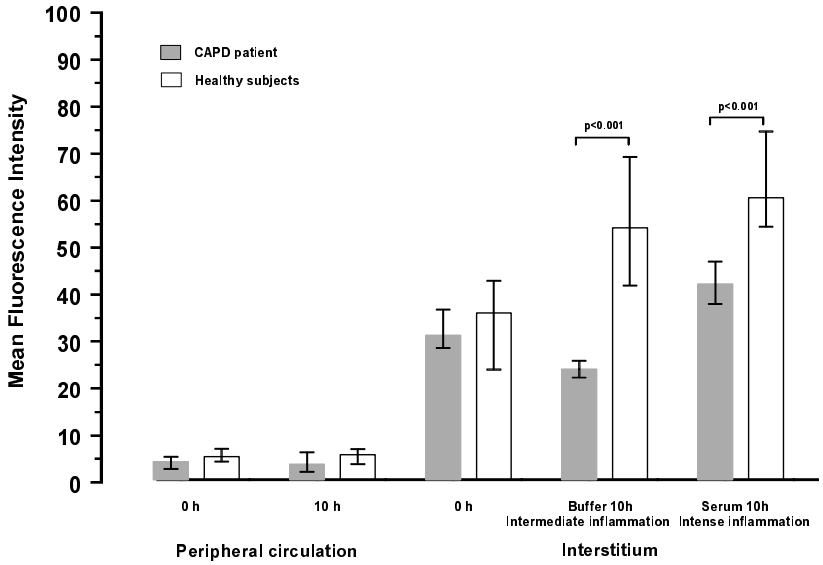

Figure 6 Expression of CD11b (median ± interquartile range) on granulocytes in the peripheral circulation and interstitium of CAPD patients and healthy subjects.

inflammatory stimulus to increase the expression of CD11b at the site of inflammation in the interstitium than corresponding cells from healthy subjects.
The CD11b expression on monocytes in the peripheral circulation was lower in CAPD patients compared to in healthy subjects. Monocytes from CAPD patients had a reduced CD11b expression at the site of intermediate inflammation compared to monocytes from healthy subjects. However, at the site of unstimulated and intense inflammation, the expression of CD11b on monocytes was similar in patients and healthy subjects.

4.3.3 The CD62L expression in peripheral and transmigrated leukocytes

The cell surface expression of CD62L was significantly lower on granulocytes and monocytes in the peripheral circulation of CAPD patients compared to cells from healthy subjects. Upon transmigration the CD62L expression decreased in both patients and healthy subjects. At the sites of intermediate and intense inflammation granulocytes from CAPD patients showed a significantly higher expression of CD62L compared to corresponding cells from healthy subjects ($p<0.001$ and $p<0.001$ respectively). No differences in the CD62L expression on monocytes at the site of interstitial inflammation was observed between CAPD patients and healthy subjects.

4.3.4 Concentration of MCP-1 and IL-8 in skin blister exudates

The concentration of MCP-1and IL-8 was reduced in blister exudates collected from sites of intermediate inflammation in CAPD patients compared to healthy subjects ($p<0.05$ and $p<0.05$ respectively). Furthermore, there were no correlations between the concentrations of MCP-1 and IL-8 in blister exudates and the expression of CD11b on monocytes and granulocytes at the sites of interstitial inflammation.

4.4 STATE OF ACTIVITY OF MONOCYTES FROM PATIENTS WITH MODERATE RENAL FAILURE (UNPUBLISHED DATA)

4.4.1 CD11b expression and response to exogenous fMLP

The expression of CD11b on peripheral and in vivo transmigrated monocytes and their responsiveness to fMLP were determined in patients with moderate renal failure (GFR 31 (27-52) mL/min). Monocytes from patients had the same capacity as cells from healthy subjects to express CD11b in the peripheral circulation and at the three different sites of inflammation.

Activation of monocytes with fMLP caused an increase in the CD11b expression both in cells in the peripheral circulation and on cells collected from the skin blisters. In patients, the fMLP mediated

CD11b expression was similar to that in healthy subjects in the peripheral circulation and at the sites with different intensities of inflammation.

4.4.2 Oxidative burst in patients with moderate renal failure

The oxidative burst in monocytes at 4ºC and after in vitro activation with fMLP and PMA was measured. In both patients and healthy subjects, incubation with fMLP induced a significant increase in oxidative burst compared to baseline. PMA stimulation caused a significantly higher H_2O_2 production in monocytes collected from the peripheral circulation compared to transmigrated monocytes. Peripheral and in vivo transmigrated monocytes from patients with impaired renal function had similar H_2O_2 production following fMLP and PMA stimulation as corresponding cells from healthy subjects.

4.5 GENE EXPRESSION IN NEUTROPHILS FROM PATIENTS WITH SEVERE RENAL FAILURE (IV)

In order to understand the molecular mechanisms that contribute to the dysfunctionality of leukocyte in patients with renal failure, gene expression profiling was performed on peripheral and in vivo transmigrated neutrophils from patients with severe renal failure (GFR<20 mL/min) and healthy subjects.

4.5.1 Gene expression during transmigration

In both patients and healthy subjects, transmigration induced a significant change in gene expression pattern. The major differences in gene expression were observed in genes involved in adhesion related activity and catalytic activity. Transmigrated neutrophils from patients and healthy subjects showed an upregulation of proinflammatory genes *MCP-1 (CCL2), MIP-1α (CCL3),* MIP-2α (CXCL2) and *MIP-3 α (CCL20*).

Transmigrated neutrophils had an upregulation of genes involved in wound healing (*laminin 5 β3*, *THBS1*and *TGFβ1*). This has also been observed by Theilgaard-Monch et al. 2004 and is related to the fact that blistering trauma itself triggers a tissue repair response.

4.5.2 Gene expression in patients with severe renal failure

In order to study the impact of renal failure on neutrophils' gene expression, we compared the gene expression of peripheral and in vivo transmigrated neutrophils from patients and healthy subjects. The majority of the differences in gene expression between cells from healthy subjects and cells from patients were observed in transmigrated neutrophils at site of interstitial inflammation.

4.5.2.1 Proinflammatory genes at the interstitial site

The leukocytes migrate from the peripheral circulation into the tissues in response to a gradient of chemotactic factors. Twenty-three genes involved in chemotaxis were differentially expressed in patients and healthy subjects. The major differences were observed at the interstitial sites. At the interstitial site, genes for chemokines critical for recruitment of additional neutrophils, T-cells and modulation of the immune response (*MIP-1α, MIP-1β, MIP-2α* and *1L-8*) were significantly upregulated in cells from patients. Furthermore there was a higher expression of genes critical for recognition of microorganisms (*FPR1*, *Complement component 3 receptors* and *GPR77* which is a receptor for C5a,C4a,C3a and their derivates) in transmigrated neutrophils from patients compared to corresponding cells from healthy subjects. Patients showed a reduced gene expression of *IL-16* both in peripheral circulation and at the interstitial site compared to healthy subjects. Furthermore, there was an increase in genes involved in tissue remodelling (*PLAU* and *PLAUR*).

4.5.2.2 Differentially regulated genes involved in adhesion molecules

At the interstitial site, patients with renal failure had a reduced gene expression of transforming growth factor type beta 1 (*TGFB1*) and thrombospondin 1 (*THBS1*), genes involved in wound healing, compared to neutrophils from healthy subjects. In contrast, transmigrated neutrophils from patients had an increased gene expression of tumor necrosis factor alpha induced protein 6 (*TNFAIP6*, also called *TSG-6*) compared to transmigrated neutrophils from healthy subjects.

5 DISCUSSION

Patients with chronic renal failure are highly susceptible to infections. One contributing factor is dysfunctional polymorphonuclear cells (Cohen G 1997, Hörl 1990). The dysfunctionality is manifested as impaired carbohydrate metabolism, reduced chemotaxis, decreased phagocytic ability and reduced intracellular killing (Muniz-Junqeira MI 2005, Lewis SL et al 1987*)*. In addition, an impaired vaccination response and a decreased IgG production by B lymphocytes, have been demonstrated (Raskova J 1987).

The majority of the studies regarding immune response in patients with renal failure have been performed on cells in the peripheral circulation, peritoneal fluid (Brauner 1998, Liberek 2004) or kidney biopsies (Temonen 1996). Apart from cells from peritoneal fluid, little is known about how leukocytes from patients with renal failure respond to inflammation at the interstitial site, where the neutrophils and monocytes confront the pathogens. To extend these observations the skin chamber technique was applied on patients with renal failure.

The skin chamber technique enables us to study local leukocyte exudation without systemic inflammatory responses. Leukocytes are recruited to the skin chambers by various chemoattractants. The choice of attractant is important, since the accumulation and activity of leukocytes in this model is highly dependent on the composition of the chamber fluid. In our studies, we have used PBS and autologous serum in order to induce an intermediate and intense inflammation. Serum serves as a potent chemoattract since it contains components from the coagulation and complement systems.

There are several in vitro methods for studying transmigration, e.g. Transwell and Boyden chambers. Although these are good methods, they do not cover all cellular events in transmigration, the way use of the skin chamber does. However, the skin chamber technique has some limitations; the target organ is less important and only a limited number of cells can be harvested from the skin window. The major technical problems are leakages and bleeding. Bleeding can cause unintentional activation of cells due to activation of the coagulation and complement systems. Leakage results in loss of cells and reduces the number of cells available for analysis.

5.1 TRANSMIGRATED MONOCYTES AND THEIR STATE OF ACTIVITY

Monocytes play a fundamental role in the immune defense against pathogens. To exert their action, monocytes migrate from the peripheral circulation into interstitial tissues where they differentiate to macrophages. The differentiation process is characterized by change in the expression of adhesion

molecules and cytokines (Ammon 2000, Andreesen 1983, Valledor 1998). In this thesis, we determined the state of activity of newly recruited monocytes with special attention focused on their role in the defense against pathogens. Two important physiological responses of monocytes are upregulation of the adhesion molecule CD11b and the ability to produce H_2O_2.

Transmigrated monocytes had an increased CD11b expression compared to peripheral monocytes. A transmigration associated upregulation of CD11b has previously been reported in neutrophils and has been related to the priming that occurs during the transmigration process (Follin 1999, Liberek 2004). In order to mimic the monocyte response towards a bacterial peptide, monocytes were stimulated with fMLP. Transmigrated monocytes had a significantly higher fMLP mediated CD11b expression compared to peripheral monocytes exposed to the same stimulus. This indicates that monocytes at the interstitial site increase their responsiveness towards fMLP. Contrary alveolar macrophages (Lundahl 1996, Haugen 1999) and atherosclerotic lesion-derived macrophages have a reduced CD11b expression (Gray JL 1995). This indicates that the ability to upregulate CD11b may be reduced when monocytes differentiate to macrophages. However, macrophages are a heterogeneous group where the local environment influences the function of macrophages (Stout 2004).

Human phagocytes eliminate microorganisms by phagocytosis. Intracellular oxygen production constitutes a key event in this process. Transmigrated monocytes had a preserved H_2O_2 production upon stimulation with fMLP, but a reduced response to PMA, compared to peripheral monocytes. The increased fMLP response can be explained by primed response against fMLP (Zimmerli 1987, Briheim 1988). In conclusion, in vivo transmigrated monocytes preserve their capacity to respond to fMLP in terms of CD11b upregulation and H_2O_2 generation.

5.1.1 Soluble factors in skin blister exudates in healthy subjects

The impact of interstitial environment on CD11b expression was studied by measuring the concentrations of the proinflammatory cytokines TNF-α, IL-6 and MCP-1, in skin blister exudates, and their correlation with CD11b. The highest concentration of cytokines was observed at the site of intense inflammation, which is in line with reports from other groups (Zweiman B 1997, Kuhns DB). We found a positive correlation between TNF-α and CD11b expression at the interstitial site. A plausible explanation for this correlation is that the CD11b integrins on monocytes transmit secondary signals that markedly enhance TNF-α expression (Fan 1993).

We conclude that in vivo transmigrated human monocytes, before differentiation to macrophages, have a preserved ability to respond to challenge with bacterial peptides in terms of CD11b

upregulation and intracellular hydrogen peroxide production. We therefore propose a role for newly recruited interstitial monocytes in the immediate response against invading pathogens.

5.2 STATE OF ACTIVITY OF MONOCYTES AND GRANULOCYTES IN PATIENTS WITH RENAL FAILURE

5.2.1 Number of transmigrated cells

The migration of leukocytes into infected or injured tissue is a fundamental step in the host-defense mechanism. Reduced leukocyte migration *in vivo* has been demonstrated in patients with diabetes mellitus (Brayton 1970) and rheumatoid arthritis (Walker 1979). We therefore investigated the capacity of leukocytes from patients with renal failure to transmigrate to the interstitial site *in vivo*. In paper I we show that monocytes from patients with severe renal failure had the same capacity as healthy subjects to transmigrate to the inflammatory site (time 0, intermediate inflammation and intense inflammation). Similar pattern have been observed in granulocytes from patients with severe renal failure (Dadfar 2004) and CAPD patients (paper II). The results indicate that renal failure has no major impact on the extravasation of leukocytes to the interstitial site in terms of accumulation rate of neutrophils and monocytes. However, our results are based on data collected at a specific time point and after a limited number of stimuli. Other time points and different stimuli might produce different results.

5.2.2 Monocyte CD11b expression in patients with renal failure

CD11b is an important adhesion molecule with a multifunctional role. The molecule is involved in the leukocyte transmigration process, and plays an important role in the innate immunity against microbes (Ehlers MRW 2000). We therefore investigated the CD11b expression in peripheral and in vivo transmigrated monocytes from patients with renal failure. Monocytes from patients with moderate renal failure had the same capacity to upregulate CD11b as those from healthy subjects.

A different pattern was observed in monocytes from patients with severe renal failure. These patients had a reduced CD11b expression on cells in the peripheral circulation compared to healthy subjects. The reduced CD11b expression was also present when cells had been recruited to the blister sites exposed to intermediate and intense inflammation. In addition, monocytes from patients with severe renal failure had a reduced ability to shed CD62L upon transmigration to the interstitial site. Monocytes from CAPD patients had a decreased expression of CD11b at the site of intermediate inflammation compared to corresponding cells from healthy subjects. At the site of intense inflammation the expression of CD11b was similar as in healthy individuals (Table 6).

Patients	Peripheral Circulation		Interstitial Site		
	Blood 0h	Blood 10h	Time 0h	Intermediate inflammation	Intense inflammation
Early renal failure	—	—	—	—	—
Severe renal failure (paper I)	⇩	⇩	—	⇩	⇩
CAPD (paper II)	⇩	⇩	—	⇩	—

Table 6. CD11b expression of monocytes from patients with renal failure compared to monocytes from healthy subjects, in peripheral circulation and at the interstitial site, studied with skin chamber technique. — = no difference in CD11b expression, ⇩ = reduced CD11b expression.

There are several possible explanations for the differences in CD11b expression on monocytes observed at the inflammatory site. One is that the cells are selectively recruited. Monocytes with high expression of CD11b may transmigrate to a larger extent. Monocytes from patients with renal failure may also have a reduced pool of intracellular CD11b or a defect in the translocation of granules and vesicles, which aggravates the upregulation of CD11b in the interstitium. In addition, the interstitial milieu may differ between patients and healthy subjects.

In order to explore whether the impaired CD11b expression observed on monocytes from patients with renal failure is dependent on the interstitial milieu, *in vitro* incubation experiments were performed. Blister exudates from patients with severe renal failure and healthy subjects had the same ability to induce CD11b expression, indicating that the interstitial milieu in patients had the same biological capacity to mobilize the CD11b receptor as that in healthy subjects. Furthermore, addition of monoclonal neutralizing antibodies towards MCP-1 had no impact on the expression of CD11b on monocytes following incubation in blister exudates *in vitro*, neither in patients nor in healthy subjects. Thus, monocytes that have transmigrated and been recruited to inflammatory sites in the interstitium in patients with renal failure have an impaired ability to mobilize the CD11b receptor to the cell surface despite the fact that cells migrate in a milieu with a similar biological capacity to mobilize the receptor.

One factor that might influence the expression of adhesion molecules in the interstitium is the concentration of leukocyte inhibitory proteins (Haag-Weber 1994, Ziesche 1994). These proteins have been reported to inhibit chemotactic movements of leukocytes towards chemoattractants during bacterial infections (Cohen 1997). They might also bind to the CD11b receptors and thereby limit their function. Another possibility is that monocytes in patients with renal failure are more refractory to further stimulation due to an altered modulation of CD11b on cells in the peripheral circulation.

We conclude that monocytes from patients with renal failure had a lower expression of CD11b despite the fact that the biological activity in terms of CD11b mobilizing factors in the interstitium was similar. We therefore propose that the impaired capacity of these monocytes to express CD11b at the interstitial site is more dependent on constitutive cellular factors than the concentration of soluble mediators.

5.2.3 CD11b expression on granulocytes in patients with renal failure

In addition to the CD11b expression of monocytes, the ability of granulocytes from CAPD patients to modulate CD11b was determined. In the peripheral circulation, granulocytes from CAPD patients had the same capacity to upregulate CD11b as those from healthy subjects. During transmigration to the unstimulated blister, the CD11b expression increased. However, granulocytes from CAPD patients were unable to upregulate CD11b to same extent as those from healthy subjects, at sites of intermediate and intense inflammation. A similar pattern has been observed in granulocytes from patients with severe renal failure (Dadfar 2004) and patients on hemodialysis (Jacobson 2000). Taken together, these data indicate that granulocytes obtained from patients with renal failure seem to require a higher intensity of inflammatory stimuli to increase the expression of CD11b at the site of interstitial inflammation. Since CD11b plays a crucial role for innate immunity to invading microbes, these phenotypic aberrations may have pathophysiological consequencesas may be manifested by the increased susceptibility to infectious diseases observed in patients with renal failure.

One possible explanation of the refractoriness of leukocytes is that the intracellular pool of CD11b might be decreased, or that there may be a defect in the translocation of granules and vesicles, which controls the upregulation of CD11b in the interstitium. It is also possible that granulocyte inhibitory proteins will accumulate in patients with CAPD. Indeed, studies have shown that immunoglobulin light chains and Granulocyte Inhibitor Protein I (GIP I) and GIP II can be isolated from peritoneal dialysis effluents (Cohen G 1995, Cohen G 1997).

5.3 GENE EXPRESSION DURING TRANSMIGRATION

Neutrophils constitute a major group of granulocytes. In order to understand the molecular mechanisms that contribute to the leukocyte dysfunction in patients with renal failure, a gene expression profiling was performed on peripheral and *in vivo* transmigrated neutrophils from patients with severe renal failure and healthy subjects. To acquire a sufficient amount of cells, three chambers were stimulated with autologous serum (representing intense inflammation).

Transmigration of neutrophils to the interstitial site was accompanied by substantial changes in gene expression. This is probably due to activation of neutrophils during the transmigration process and has been observed by another group (Thielgaard Mönch 2004). Activated neutrophils produce and release inflammatory cytokines such as IL-1, IL-8 and MIPs (Cassatella 1997) and the corresponding genes were expressed in the transmigrated neutrophils in this study. Furthermore, transmigrated neutrophils display an upregulation of genes involved in wound healing.

5.4 NEUTROPHIL GENE EXPRESSION IN PATIENTS WITH RENAL FAILURE

The major differences in gene expression between healthy subjects and patients with renal failure were observed at site of inflammation, a site at which neutrophils exert their function.

Transmigrated neutrophils from patients with renal failure had a higher expression of proinflammatory genes, such as *CCL20, IL-1a,* and *IL-8*, compared to corresponding cells from healthy subjects. The increased expression of proinflammatory genes in neutrophils from patients could partly be caused by a chronic low grade persistent inflammation. Several groups have shown that chronic inflammation in patients with renal dysfunction initiates priming of neutrophils (Sela 2005, Ward RA 1995). Monocytes from patients with renal failure have also been shown to have a higher expression of proinflammatory mediators. This "preactivation" is thought to be the result of chronic inflammation (Grindt 1995, Grindt 1998).

Transmigrated neutrophils from patients with renal failure had a higher expression of *MCP-1 (CCL2)* gene at the interstitial site of inflammation compared to corresponding cells from healthy subjects. The increased *MCP-1* gene expression may be due to activation of neutrophils, since primed and activated neutrophils express high levels of *MCP-1* mRNA (Malcolm 2003). Several studies imply that neutrophils play an important role in the activation of adaptive immunity by recruiting monocytes and T-cells (Burg 2001, Bennouna 2003). Compared to healthy subjects, patients with renal failure had a more pronounced expression of genes involved in recruitment of T-cells and DCs, among them MIP-1α (CCL3), MIP-1β (CCL4), and MIP-3α (CCL20) (Scapini 2001).

IL-8 is a proinflammatory cytokine that activates neutrophils (Martinez 2004, Wozniak 1993). Our results show that transmigrated neutrophils from patients with renal failure express increased amounts of RNA for *IL-8, IL-8RA* and *IL-8RB* compared to corresponding cells in healthy subjects. The increased *IL-8* expression was confirmed by real-time PCR and may be caused by the primed state of neutrophils (Sela 2005). However, we have previously shown, by ELISA technique, that patients with renal failure have a reduced concentration of IL-8 protein in the blister exudates compared to healthy

subjects (Dadfar 2004). In that study, the total concentration of IL-8 in skin blister exudates was measured. A plausible explanation is that the IL-8 in blister fluid comes from many other cells and not only from neutrophils: IL-8 can also be secreted by macrophages, fibroblasts and endothelial cells (Curfs 1997, Borish 2003).

Following transmigration to the site of inflammation, neutrophils from both patients and healthy subjects display an increased expression of genes involved in wound healing. This has also been observed by Theilgaard-Monch et al. and is related to the fact that blistering trauma itself triggers a tissue repair response.

However, at the interstitial site, transmigrated neutrophils from patients with renal failure showed an 8 times lower expression of transforming growth factor type beta 1 (*TGFβ1)* compared to cells from healthy subjects. TGFβ1 plays a central role in wound healing (Roberta AB 1996). The importance of TGFβ1 is observed in the TGFβ1 knockout mouse, which shows delayed wound healing (Crowe MJ 2000). Patients with renal failure also have impaired wound healing (Haag Weber 1992, Cheung AH 2001). The impaired wound healing in patients is partly a consequence of malnutrition (Haag Weber 1992). In addition to malnutrition, the downregulated *TGFB1* expression in patients may therefore contribute to the reduced wound healing in patients with renal failure.

The present study demonstrates for the first time, that neutrophils from the peripheral circulation and at the interstitial site in patients with renal failure have a different gene expression pattern compared to corresponding neutrophils from healthy subjects. The greatest differences in gene expression were observed at the interstitial site. Furthermore, transmigrated neutrophils from patients with renal failure showed a higher expression of genes encoding proinflammatory cytokines and cytokines involved in T-cell and dendritic cell recruitment compared to cells from healthy subjects.

5.4.1 Microarray

The microarray technologies enable us to study expression of thousands of genes at the same time with only a small amount of RNA. By comparing expression pathways it is possible to find defects in specific pathways, find disease-causing mechanisms and identify candidate genes for treatments. Microarray studies often deviate from the principles of conventional hypothesis-driven studies, but unexpected results from microarray studies may function as hypothesis generators instead. However, the gene array technique has its drawbacks. It is expensive, the analysis procedures are complex and it is difficult to find trends in multidimensional expression patterns. In addition, not all RNA is translated to functional proteins. The RNA may be degraded in the cells or give rise to

nonfunctional proteins. In addition, gene array does not answer the question about the functionality of the protein. The results should be further confirmed by PCR, Western blot and functional studies. However, gene array is a good platform for gene expression analysis and therefore a good starting point.

6 CONCLUSION

Monocytes are critical for both innate and adaptive immunity. To exert their action, monocytes migrate from the peripheral circulation into infected or injured interstitial tissues. Transmigration is an important process that initiates several changes in cellular function. Here we show that, in vivo transmigrated monocytes, prior to their differentiation to macrophages, have a preserved ability to respond to challenge with bacterial peptides in terms of CD11b upregulation and intracellular hydrogen peroxide production. This indicates that newly recruited monocytes have an important role in the immediate response against invading pathogens.

Patients with renal failure have dysfunctional leukocytes. Here we found that monocytes in patients with renal failure have a reduced ability to upregulate CD11b at the interstitial site compared with cells from healthy subjects, and that the reduction in CD11b expression is more dependent on cellular factors than on the concentration of soluble mediators in the interstitial milieu.

A reduced ability to upregulate CD11b at the interstitial site was also observed in monocytes and granulocyte from patients on peritoneal dialysis. The impaired CD11b expression was not dependent on the concentration of soluble IL-8 and MCP-1 in this patient group.

In addition, neutrophils from patients with renal failure have a different gene expression pattern compared to neutrophils from healthy subjects, both in the peripheral circulation and at the interstitial site. The greatest differences were observed at the interstitial sites. Patients with renal failure had a higher expression of proinflammatory cytokines and cytokines involved in T-cell and dendritic cell recruitment.

Taken together, these results contribute to an increased understanding of the factors involved in the impaired immune response observed in patients with renal failure.

7 ACKNOWLEDGMENTS

I wish to express my sincere gratitude to all those who had helped, supported and encouraged me during these years. In particular, I would like to thank:

Joachim Lundahl, my supervisor, for sharing your profound knowledge in immunology. For never ending inspiration, enthusiasm and interest in research. Thanks for helping me finding solutions when I had reached a dead end.

Stefan H Jacobson, my co-supervisor, for constant support, your effectiveness, and for your constructive criticism of my papers, I've learned a lot.

To Jocke and Stefan I would like to say "ett stort TACK".

Marianne Van Hage, head of Clinical Immunology & Allergy Unit, for providing excellent working conditions.

My co-authors **Eva Fernvik, Anna Nopp, Britta Hylander, Jenny K Olsson** and **Josefin Paulsson** for pleasant and good co-operation.

SGO Johansson for your help and support, especially in the beginning of my PhD studies.

Anneli Brauner and **Magnus Sköld** for pleasant and fruitful collaborations.

Hojjat Rabbani for introducing me in the field of Real-time PCR.

To all the former and present member of the "Inflammatory Unit" **Katarina Forsberg, Cecilia Hellman, Ali Moshfegh, Anna Nopp, Titti Nieminen, Jenny Olsson, Josefin Paulsson, Lena Wehlin, Agneta Wikman** and **Elham Yektaie**. For support during these years and sharing all the good and bad times as a PhD student. Thanks for all inspiring and lovely discussion about science, life, travel and home decorating and for all nice travel memories (I'm still afraid of height although some of you tried to cure it!). With you around there is no dull moment in the lab.

Anett Bygdén (**Nessie**), our angel. Thanks for coming in during the weekends and provide me with samples, so I could continue with my research while I was studying and thanks for all "det fixar jag".

To always smiling **Gerd Franzon-Lundqvist** for your support with administration. Thanks for always finding a clever solution.

All my friends and colleagues at "**plan 4**" and "**lungforskningslabb**" for all your support (antibodies, computer, cell sorter and dendritic cells) and for all the nice "chats" during lunch and coffee breaks. Thanks for creating a friendly and inspiring atmosphere.

My former classmates in Uppsala, specially **Susanne, Eva, Victoria, Camilla** and **Erik**, for all the good tip and advices.

Yvonne & Ivar, close but far away friends. Thanks for all the long phone calls with "briefing" about life and research. Your advices are irreparable. I wish you both, good luck in the future.

My friends: **Shiva, Peiman, Ghazal, Shahram, Kia, Homaion, Parisa, Aptin, Jenny** and **Shadi,** Thanks for all the fun and enjoyable dinners and all the alternative carrier suggestions!

My mother in law "**madarjon**" for all your support and fabulous food.

My parents for always being there and believing in me. Thanks for your never ending love, for all your advice and support. My **sister Anna** and my **brother Ali** for sharing many happy moments, love and encouragement.

Ali (Navid), my love, thanks for your endless support and for standing by my side in life's roller coaster. I hope you know how much you mean to me.

Elham Dadfar
Stockholm 2006-04-08

This study was also supported by unrestricted grants from the Karolinska Institutet, the Swedish National Federation of Kidney Patients, the Swedish Society of Nephrology, the Lisa and Johan Grönbergs Stiftelse and TERUMO EUROPÉ N.V., dedicated to *well being*.

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