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**LEUKOCYTE DYSFUNCTION AND
INFLAMMATORY MARKERS
IN PATIENTS WITH
CHRONIC KIDNEY DISEASE AND
PATIENTS ON DIALYSIS**

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To my family and friends

*We ourselves feel that what we are doing
is just a drop in the ocean. But the ocean
would be less because of that missing drop.*
Mother Teresa

ABSTRACT

Patients with chronic kidney disease (CKD) and patients on dialysis are at risk for serious infectious complications. This is partly due to leukocyte dysfunction, which is a consequence of both uremic retention solutes and of dialysis.

Paper I describes functions of *in vivo* extravasated monocytes and neutrophils (CD11b/CD18 expression, respiratory burst and apoptosis) from patients on high-flux hemodialysis/hemodiafiltration (HD/HDF) and healthy subjects. In contrast to our previous observations in patients on low-flux HD, CKD and peritoneal dialysis, we found similar mobilization of CD11b/CD18 and also in the apoptotic rate in patients on high-flux HD/HDF and in healthy subjects, which can be interpreted as a preserved leukocyte function in this dialysis population. CD11b/CD18 is an important adhesion molecule for leukocyte transmigration and phagocytosis. There were differences in the respiratory burst between patients and healthy subject, which could be due to leukocyte refractoriness when challenged with a strong inflammatory stimulus.

Paper II describes the concentrations of important chemokines for neutrophils (IL-8 and MMP-9/NGAL) and monocytes (MCP-1 and MIP-1 α) in the peripheral circulation and at the site of interstitial inflammation in patients on high-flux HD/HDF, compared with healthy subjects. We found similar (IL-8, MMP-9/NGAL and MIP-1 α) or even higher (MCP-1) concentrations of these chemokines at the site of interstitial inflammation in patients on high-flux HD/HDF compared with healthy subjects. One of the mechanisms for the preserved leukocyte function demonstrated in Paper I could be an equal production of chemokines at the site of inflammation, which has not been demonstrated for patients with CKD or patients on low-flux HD.

Paper III describes the CD11b/CD18 up-regulation and gene transcription of TGF β , CD40, IRAK-1, IL8 and IL12A by *in vitro* LPS stimulation and *in vivo* extravasation of neutrophils. We found a similar fold change in gene transcription of TGF β , CD40 and IRAK-1, as well as a similar range of CD11b/CD18 mobilization by LPS stimulation and extravasation, indicating a potential use of *in vitro* LPS stimulation as a model for studying *in vivo* activation of neutrophils.

Paper IV describes the transcriptional regulation after LPS stimulation of neutrophils from patients with CKD stage 3-5 and healthy subjects. In patients with CKD, there is a weak LPS-mediated up-regulation or even a down-regulation of important proinflammatory mediators, among these SOD2, whereas in healthy subjects there is a strong up-regulation. SOD2 is of central importance for both the up-regulation of other proinflammatory mediators and neutrophil respiratory burst, which is demonstrated by inhibition of SOD2 in differentiated HL60 cells and subsequent analysis of gene transcription fold change and respiratory burst after PMA stimulation. The impaired up-regulation of SOD2 following LPS stimulation could be one of the mechanisms responsible for neutrophil dysfunction observed in CKD patients.

LIST OF PUBLICATIONS

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stimulation: a potential cause of neutrophil dysfunction in chronic kidney
disease.
Submitted manuscript.

CONTENTS

1	Introduction	1
1.1	Neutrophils and monocytes.....	1
1.1.1	Adhesion molecules	2
1.1.2	Selectins and integrins	3
1.1.3	Leukocyte extravasation and transmigration	4
1.1.4	Respiratory burst	5
1.1.5	Apoptosis	6
1.2	Chemokines and inflammatory markers	6
1.3	LPS signaling pathways.....	8
1.4	Patients with chronic kidney disease	10
1.4.1	Leukocyte dysfunction in CKD	10
1.4.2	Patients on HD	11
1.4.3	Low-flux and high-flux HD and convective therapies	13
1.5	Gene transcription in CKD	15
1.5.1	The IL-1 family.....	15
1.5.2	IL-1 receptors.....	16
1.5.3	Superoxide dismutase 2 (SOD2).....	17
1.5.4	Other markers.....	17
1.6	The HL60 cells.....	18
1.7	Oxidative stress and endothelial dysfunction.....	18
2	Aims.....	20
3	Methods	21
3.1	Patient characteristics.....	21
3.2	The skin chamber method for <i>in vivo</i> extravasation	22
3.3	Analyses by flow cytometry	23
3.3.1	Cell counts	24
3.3.2	CD11b/CD18 expression	24
3.3.3	Respiratory burst.....	24
3.3.4	Apoptosis	24
3.4	Chemokines in blister fluid and in the peripheral circulation	25
3.5	Neutrophil isolation and <i>in vitro</i> LPS stimulation	25
3.6	HL60 cell differentiation and inhibition of SOD2 and measurement of respiratory burst.....	25
3.7	RNA extraction and quantitative PCR.....	25
4	Statistical analysis.....	26
4.1	Paper [I-II].....	26
4.2	Paper [III-IV]	26
5	Results and discussion.....	27
5.1	CD11b/CD18 expression on leukocytes	27
5.2	Respiratory burst and apoptosis	27
5.3	Chemokines.....	30
5.4	<i>In vitro</i> LPS-activated neutrophils	32
5.5	Inhibition of SOD2 and respiratory burst	33

	5.6	<i>In vivo</i> extravasation versus <i>in vitro</i> LPS stimulation regarding CD11b/CD18 up-regulation and gene transcription	35
6		Conclusions	36
7		Future perspectives.....	37
8		Acknowledgements	38
9		References	40

LIST OF ABBREVIATIONS

CD	Cluster of differentiation
CKD	Chronic kidney disease
CRP	C-reactive protein
DCFH-DA	Dichlorofluorescein diacetate
ELAM	Endothelial cell leukocyte adhesion molecule
ELISA	Enzyme-linked immunosorbent assay
ESRD	End-stage renal disease
FACS	Fluorescence-activated cell sorting
GFR	Glomerular filtration rate
fMLP	N-formylmethionyl leucyl phenylalanine
HD	Hemodialysis
HDF	Hemodiafiltration
HDL	High-density lipoprotein
HF	Hemofiltration
ICAM	Intercellular cell adhesion molecule
IL	Interleukin
IL-1Ra	IL-1 receptor antagonist
ICE	IL-1 converting enzyme
IFN	Interferon
IL8RA	IL-8 receptor α gene
IRAK	IL-1 receptor-associated kinase
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
MCP	Monocyte chemotactic protein
MFI	Mean fluorescence intensity
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinase
MyD88	Myeloid differentiation primary response gene 88
NADPH	Reduced form of nicotinamide adenine dinucleotide phosphate
NF κ B	Nuclear factor κ B
NGAL	Neutrophil gelatinase-associated lipocalin
NS	Nonsignificant
PADGEM	Platelet activation dependent granule-external membrane protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PECAM	Platelet-endothelial cell adhesion molecule
PI	Propidium iodide
PMA	Phorbol-12-myristate-7-acetate
PS	Phosphatidyl serine
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TGF	Transforming growth factor
TLR	Toll-like receptor
TNF	Tumor necrosis factor

TRAF
VCAM
VLA

TNF receptor-associated factor
Vascular cell adhesion molecule
Very late antigen

1 INTRODUCTION

Patients with chronic kidney disease (CKD) and patients on renal replacement therapy such as hemodialysis (HD) and peritoneal dialysis (PD) have an increased susceptibility to infectious diseases compared with healthy subjects (Sarnak and Jaber 2000; Allon, Depner et al. 2003). Infection is also the second most common cause of morbidity and mortality in patients with end stage renal disease (Bloembergen and Port 1996; Powe, Jaar et al. 1999; Graff, Franklin et al. 2002). Contributing factors could be the chronic inflammatory activation seen in CKD patients and patients on dialysis, which causes a refractoriness of leukocytes when confronted with invading microorganisms.

1.1 NEUTROPHILS AND MONOCYTES

The immune system is designed to defend us from invading microorganisms, such as viruses and bacteria. The first response is called the innate immune response, mostly dependent on recruitment and activation of neutrophils (Parkin and Cohen 2001). Complement activation occurs on the bacterial cell surface and this activates a cascade of proteolytic reactions that are specific in so far as they act on microbial surfaces but not on host cells. Neutrophils have receptors both for common bacterial constituents and for complement. Neutrophils become activated through complement (C3b and C5a) but can also get activated directly by bacterial peptides, such as lipopolysaccharide (LPS), lipotechoic acid, mannans and fMLP (N-formylmethionyl leucyl phenylalanine) (Parkin and Cohen 2001). Neutrophils exist in the circulation in different states of activation. Activation of neutrophils occurs in two steps. At the first step, the neutrophil gets primed by an activating signal, and at the second step, the neutrophil is further activated to reach the site of inflammation and perform its specific action: phagocytosis and release of inflammatory mediators (Swain, Rohn et al. 2002).

Neutrophils are key effector cells in the innate immunity, and an impaired neutrophil function has many negative consequences for the defense against invading microorganisms.

Neutrophils have generally been regarded as fully differentiated cells whose function is based on preformed receptors and soluble factors. This view has recently been challenged by publications that suggest a second gene transcriptional

activity following extravasation (Theilgaard-Monch, Porse et al. 2006). The transcriptional activation occurs at the inflammatory site and engages genes involved in a variety of neutrophil functions, such as production of reactive oxygen species (ROS), cytokines and chemokines (Theilgaard-Monch, Knudsen et al. 2004; Coldren, Nick et al. 2006). Neutrophils direct both the innate and adaptive immune responses, by communicating with immune modulating cells (Yamashiro, Kamohara et al. 2001; Theilgaard-Monch, Knudsen et al. 2004). Neutrophil cytokine and chemokine production can be an important link between the innate and the adaptive immune response, since cytokine-primed neutrophils are able to express monocyte chemoattractant protein-1 (MCP-1/CCL2), and MCP-1 in its turn acts as a chemotactic signal for mononuclear cells and for expression of other cell surface molecules (Yamashiro, Kamohara et al. 2001). Activated neutrophils produce and release a number of proinflammatory cytokines and chemokines, including IL-1, IL-8, MCP-1 and macrophage inflammatory protein-1 α and 1 β (MIP-1 α /MIP-1 β) (Yamashiro, Kamohara et al. 2001; Kobayashi 2008). This chemokine release results in recruitment of other inflammatory cells, such as monocytes, in a subsequent step of the innate immune response.

Chemokines attract neutrophils and monocytes from the blood stream to the site of inflammation or infection by first making the endothelium more adhesive to the circulating cells and then through a chemokine gradient through the tissue, with higher concentrations of chemokines closest to the site of inflammation (Janeway and Travers 2005). Circulating monocytes that extravasate and get activated are rapidly developed to mature macrophages (Janeway and Travers 2005).

1.1.1 Adhesion molecules

The accumulation of monocytes and neutrophils at sites of infection and inflammation is a fundamental step in the host defense against invading microorganisms. The process of extravasation into inflamed tissue requires the expression of adhesion molecules on the endothelium to initiate the leukocyte adherence event (Johnson-Leger, Aurrand-Lions et al. 2000). The main families of adhesion molecules are the intercellular adhesion molecules, integrins, selectins and cadherins (calcium-dependent adherins) (Parkin and Cohen 2001).

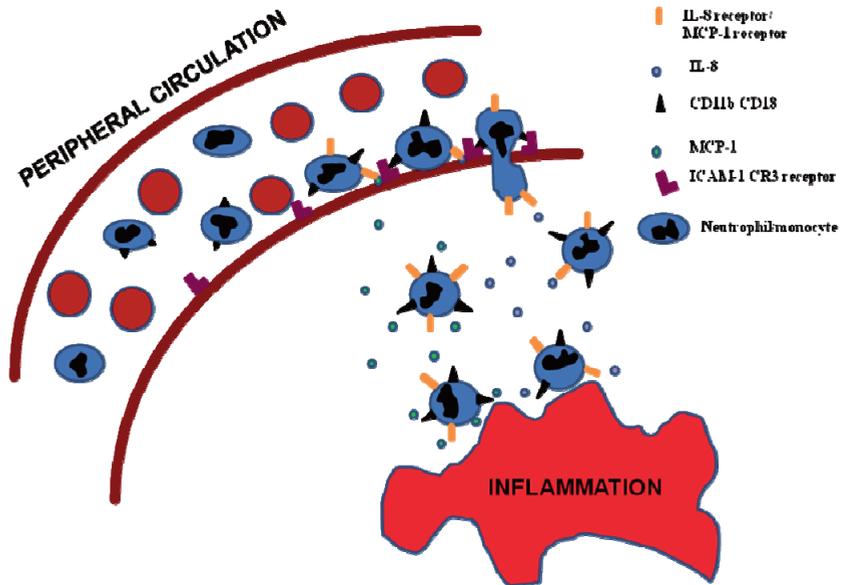


Figure 1. Leukocyte adhesion to the endothelium, subsequent extravasation and transmigration through a chemotactic gradient in the interstitium towards a site of inflammation.

1.1.2 Selectins and integrins

The recruitment of circulating leukocytes into inflamed tissues depends on interaction between adhesion molecules on leukocytes and vascular endothelial cells (van Buul and Hordijk 2004). Tethering, which is mediated by selectins, brings the leukocyte into transient contact with the vessel wall, allowing it to search the endothelium for the presence of factors that activate a secondary phase of integrin-mediated adhesion, before diapedesis into the extracellular matrix (Albelda, Smith et al. 1994).

The selectins P-selectin (PADGEM, CD62P) and E-selectin (ELAM-1, CD62E), are membrane glycoproteins with a distal lectin-like domain that binds to specific carbohydrate groups on cells. Selectins are induced on the cytokine-activated endothelium and initiate interactions between leukocytes and endothelial cells by binding to oligosaccharide ligands on passing leukocytes. CD62L is a selectin which is present on circulating leukocytes (Janeway and Travers 2005).

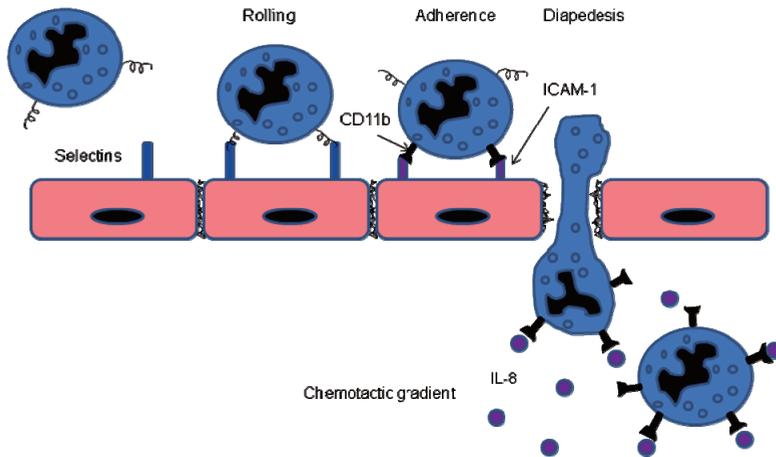


Figure 2. Neutrophil adhesion, extravasation and transmigration.

Leukocyte functional antigens LFA-1 (CD11a/CD18), LFA-2 and LFA-3 are expressed on leukocytes. They act as cell adhesion molecules (Janeway and Travers 2005) and bind to selectins on the endothelium. Intercellular adhesion molecules (ICAM-1 on resting endothelium and ICAM-2 on activated endothelium) confer tighter adhesion, due to binding of β_2 -integrins - CD11a/CD18 (LFA-1) and CD11b/CD18 (Mac-1 or CR3) - expressed on leukocytes after a chemokine-mediated conformational change in the integrins (Adams and Shaw 1994; Gonzalez-Amaro and Sanchez-Madrid 1999; Janeway and Travers 2005).

The β_1 - integrin very late antigen-4 (VLA-4) is present principally on mononuclear cells, mediating monocyte transmigration by binding to vascular adhesion molecule (VCAM-1) on activated endothelial cells (Chuluyan and Issekutz 1993).

1.1.3 Leukocyte extravasation and transmigration

Chemokines are small, structurally related molecules that interact with 7-transmembrane-spanning G-protein-coupled receptors. They contribute to leukocyte recruitment firstly by activating integrins and secondly by promoting the migration of adherent leukocytes across the endothelium and through the extracellular matrix (Adams and Shaw 1994).

Leukocyte binding to endothelium induces NADPH oxidase in the endothelial cells, promoting production of reactive oxygen species and signaling, to facilitate

leukocyte passage through the endothelial cells and through the basement membrane (van Buul and Hordijk 2004). PECAM-1 plays an important role in transendothelial migration of leukocytes, by inducing phosphorylation of tyrosine in junctional proteins which leads to loss of cell-cell adhesion (van Buul and Hordijk 2004).

When neutrophils extravasate, they produce enzymes that break down both the endothelial cell-cell junction and extracellular matrix proteins (i.e. elastase and other proteases such as matrix metalloproteinase-9, MMP-9) (Hermant, Bibert et al. 2003).

The final step of the transmigration is the attraction force of a chemokine concentration gradient through the interstitium where CXCL8 (IL-8) and CCL2 (MCP-1) act as chemotactic factors for neutrophils and monocytes, respectively. They bind to proteoglycans in the extracellular matrix and to similar molecules on the leukocytes (Janeway and Travers 2005).

Neutrophils and monocytes in blood normally express a low amount of CD11b/CD18 on their surface, containing the molecules in small vesicles of the cytoplasm. Following activation of the cells, CD11b/CD18 is transported to and expressed on the cell surface and the molecules are activated in order to exert their function (Adams and Shaw 1994; Albelda, Smith et al. 1994; Adams and Lloyd 1997; Gonzalez-Amaro and Sanchez-Madrid 1999). Mobilization of CD11b/CD18 is important in the process of leukocyte transmigration, phagocytosis and complement activation as a response to an inflammatory stimulus in the interstitium (Bainton, Miller et al. 1987; Borregaard, Miller et al. 1987; Miller, Bainton et al. 1987).

1.1.4 Respiratory burst

Phagocytes, such as neutrophils and monocytes, produce reactive oxygen species (ROS) as part of the host defense against invading bacteria. These cells generate ROS using an enzyme complex, the NADPH oxidase, in a process referred to as the respiratory burst. Respiratory burst is a central process for digestion and elimination of invading microorganisms in leukocytes (Babior 1999). Superoxide anion, generated by NADPH oxidase, is converted to hydrogen peroxide in the phagolysosome by the action of SOD2 and the catalyzing effect of catalase and glutathione peroxidase (Dahlgren and Karlsson 1999). In the absence of SOD2,

superoxide anions can form the highly aggressive oxidative substance peroxynitrite (by reacting with nitric oxide), myeloperoxidase (MPO) located in the azurophil granules, and hydroxyl radicals (Dahlgren and Karlsson 1999).

1.1.5 Apoptosis

In early apoptosis, there is a reconfiguration of the cell membrane, with phosphatidyl serine (PS) translocated from the inner surface to the outer leaflet of the cell membrane. Fluorescein (FITC) conjugated Annexin V binds to PS with high affinity and identifies early apoptotic cells. Propidium iodide enters through a damaged cell membrane after loss of membrane integrity and stains DNA, identifying late stages of apoptosis and secondary necrotic cells. PS is identified by phagocytes in the extracellular milieu in order to remove the dying cells by phagocytosis.

1.2 CHEMOKINES AND INFLAMMATORY MARKERS

Chemokines are small molecules, divided into CXC (α -chemokines) and CC (β -chemokines) depending on the position of two cysteine residues (C) with other amino acids (X) (Charo and Ransohoff 2006). Chemokines are produced by most cells after stimulation with proinflammatory cytokines or bacterial products, and there are both soluble and membrane-bound chemokines (Parkin and Cohen 2001). The chemokines and cytokines studied within this thesis are shown in Table 1 and Table 2.

Chemokines	Receptor	Functions	References	
IL-8 (interleukin-8)	CXCL8	IL-8 receptor α and β	Induces neutrophil CD11b/CD18 up-regulation, transmigration and activation. Stimulates the release of MMP-9/NGAL. Binding to the receptor causes a reformation of integrins, which allows neutrophils to bind to the endothelial cells.	(Zeilhofer and Schorr 2000; Drost and MacNee 2002; Adams and Lloyd 1997)
MCP-1 (monocyte chemotactic protein-1)	CCL2	CCR2	Chemotactic factor and activator of monocytes and macrophages. Produced by many different inflammatory cells. Induces up-regulation of CD11b/CD18 and facilitates monocyte adhesion to endothelial cells. Associated with chronic and acute inflammation, as well as the acute coronary syndrome.	(Adams and Lloyd 1997; Jiang, Beller et al. 1992; Jiang, Zhu et al. 1994; Ikeda, Matsui et al. 2002; de Lemos, Morrow et al. 2003; Pawlak, Naumnik et al. 2004)
MIP-1α (macrophage inflammatory protein-1α)	CCL3	CCR1 and CCR5	Released from monocytes after triggering of CD11b/CD18. Promotes the recruitment of inflammatory cells. Chemotactic factor for both monocytes and neutrophils. Activates macrophages by up-regulation of CD11b/CD18.	(Rezzonico, Imbert et al. 2001; Adams and Lloyd 1997; Ramos, Canetti et al. 2005; Weber, Belge et al. 2000)
MMP-9/NGAL (matrix metalloproteinase-9 in complex with neutrophil gelatinase-associated lipocalin)			MMP-9 and proteolytic enzymes degrade the extracellular matrix and promote leukocyte transmigration. Marker of neutrophil activation and release of reactive oxygen species (ROS). By cleaving of chemokines and cytokines, it regulates chemokine activity.	(Yan, Borregaard et al. 2001; Alberts, Johnson et al. 2002; Brogden and Guthmiller 2002; Van Den Steen, Wuyts et al. 2003)

Table 1. Characteristics of chemokines studied in paper II.

Cytokines	Functions	References
TNFα (tumor necrosis factor α)	Produced by macrophages and monocytes in acute and chronic inflammation. Pro-apoptotic. Up-regulates adhesion molecules on endothelial cells. Chemotactic factor for monocytes and primes cells for phagocytosis. Increases vascular permeability and vasodilatation, promotes intravascular coagulation, and causes the septic syndrome and failure of vital organs.	(Idriss and Naismith 2000; Janeway and Travers 2005)
IL-6	Inflammatory marker, important role in acute inflammation and production of acute phase proteins from the liver. Higher in ESRD patients, predicts mortality in ESRD patients starting dialysis.	(Adams and Lloyd 1997; Pupim, Himmelfarb et al. 2004; Pecoits-Filho, Barany et al. 2002; Panichi, Maggiore et al. 2004)

Table 2. Characteristics of cytokines (unpublished data) from paper II.

1.3 LPS SIGNALING PATHWAYS

Lipopolysaccharide (LPS) is a component of the outer membrane of Gram-negative bacteria and an important activator of neutrophils through interaction with LPS-binding molecules on the cell surface.

The principal plasma protein responsible for transporting LPS in the circulation is the LPS-binding protein (LBP). LPS can trigger major infectious complications, e.g. septic shock and disseminated intravascular coagulation (Dobrovolskaia and Vogel 2002; Matsuda and Hattori 2006), by induction of the three pathways of the complement system, but also through induction of proinflammatory cytokines (Chaby 2004). The plasma level of LPS and LBP has prognostic implications for patients with severe sepsis (Opal, Scannon et al. 1999). LPS binds to various proteins on the cell surface, as well as proteins released from the granules of activated neutrophils, and contributes to the progress of the inflammatory reaction by promoting release of proinflammatory cytokines and chemokines (Opal, Scannon et al. 1999; Dobrovolskaia and Vogel 2002).

CD14 exists in a soluble form and a membrane-bound form expressed on the surface of neutrophils and other inflammatory cells. The LPS-CD14 interaction plays an important role in the activation of these cells. For further activation of neutrophils additional cell membrane molecules are required, such as the Toll-like receptor 4 (TLR-4) and adaptor proteins (e.g. MD-2) (Dobrovolskaia and Vogel 2002).

TLRs form part of the pattern recognition receptors (PRR) that recognize specific bacterial and viral constituents on cell surfaces, such as LPS, bacterial lipoproteins, viral RNA or DNA. Binding to PRRs gives rise to an intracellular cascade of signaling and transcriptional regulation. The pathogen-recognition innate immune response is linked to the antigen-specific adaptive immune response by this transcriptional regulation (Iwasaki and Medzhitov). The investigation regarding PRRs that started about twenty years ago is a great field for new discoveries, since it has not yet been elucidated how these mechanisms cooperate and how the link between the innate and adaptive immune responses works.

LPS interaction with TLR-4 activates an intracellular signaling pathway that involves an adaptor protein, myeloid differentiation primary response gene 88 (MyD88), IL-1 receptor-associated kinase 1 (IRAK-1) and TNF receptor-associated factor 6 (TRAF6) (Means, Golenbock et al. 2000). A specific LPS-binding site has been shown to exist on CD11b/CD18 and CD62L, triggering LPS signaling through NF κ B (Dobrovolskaia and Vogel 2002).

Internalization of LPS into the cytoplasm of neutrophils plays an essential role in the integrin-mediated adhesion of neutrophils (Chaby 2004) and can also work as a neutralizing mechanism against the deleterious effects of LPS in the organism (Matsuda and Hattori 2006).

LPS indirectly activates the oxidative response of neutrophils by priming them for further activation by other stimuli, such as fMLP. Priming with LPS and subsequent activation by fMLP activates the NADPH oxidase and this mechanism starts the respiratory burst (Almkvist, Faldt et al. 2001). LPS priming causes mobilization of gelatinase granules and specific granules in the cytoplasm, and CD11b/CD18 is also mobilized to the cell surface by this priming (Almkvist, Faldt et al. 2001). Both LPS activation and extravasation results in priming of the cells

to a hyperresponsiveness to a second stimulus e.g. fMLP and other bacterial peptides (Guthrie, McPhail et al. 1984; Almkvist, Faldt et al. 2001).

Dialysis with bioincompatible cellulosic membranes can induce up-regulation of CD14 on leukocytes, promoting the response of these cells to stimulation with LPS (Carracedo, Ramirez et al. 1995; Carracedo, Ramirez et al. 2002). However, a constant stimulation with LPS and other bacterial toxins can also create a state of endotoxin tolerance, which makes neutrophils and other inflammatory cells refractory to further stimulation. This is likely to depend on mechanisms of early steps in LPS-mediated signal transduction, after binding of LPS to the receptor (Dobrovolskaia and Vogel 2002).

1.4 PATIENTS WITH CHRONIC KIDNEY DISEASE

CKD is staged according to the National Kidney Foundation (NKF) KDOQI guidelines in 5 stages:

CKD stage 1 GFR > 90 ml/min with other signs of chronic kidney disease

CKD stage 2 GFR 60-89 ml/min

CKD stage 3 GFR 30-59 ml/min

CKD stage 4 GFR 15-29 ml/min

CKD stage 5 GFR < 15 ml/min

1.4.1 Leukocyte dysfunction in CKD

The leukocyte dysfunction seen in CKD is complex. Contributing factors are metabolic and functional abnormalities of leukocytes caused by accumulation of uremic toxins that inhibit leukocyte function and bioincompatibility of the dialysis procedure (Lundberg, Johansson et al. 1994; Vanholder, Van Loo et al. 1996; Cohen, Rudnicki et al. 2001; Horl 2001; Cohen, Rudnicki et al. 2003; Cheung, Greene et al. 2008). In CKD, there is an altered leukocyte adherence to endothelial cells, decreased activation of inflammatory cells, impaired phagocytosis and chemotaxis, altered generation of ROS and an impaired intracellular killing of bacteria (Gibbons, Martinez et al. 1990; Haag-Weber and Horl 1996; Horl 2001). There is evidence for cytokine dysregulation in CKD, giving rise to a state of persistently activated immune system (Descamps-Latscha 1993; Malaponte, Libra et al. 2007; Carrero, Yilmaz et al. 2008).

The underlying condition of the patient, such as malnutrition, CKD *per se* and other chronic diseases, also plays an important role in this non-physiological inflammatory activity (Cohen, Haag-Weber et al. 1997; Stenvinkel, Heimbürger et al. 2000; Pecoits-Filho, Lindholm et al. 2002).

There are several uremic toxins that inhibit neutrophil functions, e.g. p-cresol, guanidino compounds, granulocyte inhibitory protein I and II, degranulation inhibitory protein I and II (identified as angiogenin and complement factor D), κ - and λ -light chains and chemotaxis inhibitory protein (Vanholder, De Smet et al. 1994; Haag-Weber and Horl 1996; Kaysen 2001; Horl 2002; Kaysen and Kumar 2003; Cohen and Horl 2009; Cohen and Horl 2009). P-cresol can impair the endothelial response to the activity of proinflammatory cytokines, with decreased expression of ICAM-1 and VCAM-1 (Dou, Cerini et al. 2002) but dialysis can also reduce leukocyte-endothelial interactions and impair transmigration (Thylen, Fernvik et al. 1997).

A study from our group has demonstrated that neutrophils and monocytes from patients with advanced CKD have an impaired expression of CD11b/CD18 in the interstitium compared with corresponding cells from healthy subjects (Dadfar, Lundahl et al. 2004; Dadfar, Lundahl et al. 2004). The same result has been shown for patients on peritoneal dialysis (Dadfar, Lundahl et al. 2004).

1.4.2 Patients on HD

When considered from a historical point of view, dialysis has saved many lives over the years; however, the life quality of patients on dialysis still has to be improved to make it an optimal treatment. In spite of the developments in the last years towards more biocompatible materials and methods, patients on hemodialysis still have a high morbidity and mortality in infections (Bloembergen and Port 1996; Powe, Jaar et al. 1999; Graff, Franklin et al. 2002).

In patients on hemodialysis, there is evidence of a chronic inflammatory activation, which could be due to several factors (Cheung, Parker et al. 1989; Haag-Weber, Hable et al. 1991; Descamps-Latscha 1993; Schindler, Eichert et al. 2001; Carracedo, Ramirez et al. 2002; Kosch, Levers et al. 2003; Koller, Hochegger et al. 2004). Small fragments of bacterial products with cytokine-inducing capacity can be present in small amounts in the dialysate and enter the circulation through the dialysis membrane (Horl 2002). Besides transfer of

bacterial substances through the dialysis membrane, which activates cytokine production (IL-6, TNF α and IL-1), direct activation of complement factors and of leukocytes by contact with the dialysis membrane are also important mechanisms for the unphysiological inflammatory activity seen in HD patients. There is also a removal of cytokines, other inflammatory mediators (LPS fragments, granulocyte inhibitory proteins 1 and 2, IL-1, TNF α) and complement factors (C3a, C5a) by the HD procedure as well as adsorption of substances to the hydrophobic high-flux membrane (e.g. factor D) (Clark, Hamburger et al. 1999; Schindler, Ertl et al. 2006). Biocompatibility of dialysis membranes probably plays an important role in determining leukocyte function in patients on HD (Himmelfarb, Lazarus et al. 1991; Himmelfarb, Ault et al. 1993; Hernandez, Galan et al. 2004; Schindler, Ertl et al. 2006).

High serum levels of cytokines and chemokines have been observed in patients on HD treatment with modified cellulose membranes (Descamps-Latscha 1993; Pawlak, Naumnik et al. 2004; Muniz-Junqueira, Braga Lopes et al. 2005). High-flux HD causes lower levels of IL-6 and IL-1 β than low-flux HD or dialysis with cuprophane membranes (Schindler, Ertl et al. 2006). In patients on HD with cuprophane or polysulfone membranes, a significantly higher serum level of MCP-1 is seen compared with healthy subjects both before and after the HD session, independent of the membrane used (Jacobson, Thylen et al. 2000; Thylen, Lundahl et al. 2000).

Neutrophil dysfunction in dialysis patients is manifested by reduced chemotaxis, adherence, respiratory burst and glucose consumption in response to an inflammatory stimulus (Vanholder, Dell'Aquila et al. 1993; Vanholder, Van Biesen et al. 1993).

Our group has previously demonstrated that neutrophils and monocytes recruited to an interstitial inflammatory site in patients treated with low-flux bioincompatible HD have an impaired capacity of mobilizing CD11b/CD18 as a response to an induced intermediate inflammation in the interstitium compared with corresponding cells from healthy subjects (Thylen, Lundahl et al. 2000; Jacobson, Thylen et al. 2002). CD11b/CD18 is important for leukocyte function in terms of phagocytosis and an impaired response might contribute to an increased susceptibility to infections observed in this group of patients.

High-flux polysulfone dialysis, as opposed to low-flux polysulfone and cuprophane treatment, has been shown to improve the transmigration of circulating neutrophils (Moshfegh, Jacobson et al. 2002). High-flux dialysis membranes decrease the levels of the two degranulation inhibitory proteins (angiogenin and complement factor D), which could contribute to the maintained respiratory burst and phagocytic capacity seen in patients on high-flux HD (Horl 2002).

The degree of spontaneous apoptosis of leukocytes is higher when bioincompatible membranes are used for HD, than when biocompatible membranes are used (Martin-Malo, Carracedo et al. 2000). This higher apoptotic activity in leukocytes is probably due to an antibody-dependent activation of the complement system caused by the material or structure of the dialysis filters. It has been shown that heat inactivation of complement components results in significantly lower apoptosis rates and that bioincompatible membranes cause a higher degree of apoptosis than biocompatible membranes (Koller, Hochegger et al. 2004).

1.4.3 Low-flux and high-flux HD and convective therapies

The HD procedure has developed greatly from the first treatment to the dialysis methods used nowadays. Dialysis started as the simple removal of uremic toxins by slow diffusion through a dialysis membrane, and has led to the development of HD membranes and the more biocompatible materials of today. Much focus has been given to convective therapies, such as hemofiltration (HF) and hemodiafiltration (HDF), and also to high-flux HD. There is now a growing interest in development of HD membranes towards more biocompatible materials, and the mechanisms of membrane flux to improve the efficiency of single HD treatments in removal of uremic retention solutes.

Middle-sized molecules are primarily cleared by diffusion, but convective therapies significantly enhance the clearance of these molecules (Clark, Hamburger et al. 1999).

Postdilution HF was the first convective therapy used, and this method provides a high clearance of middle- and large-sized molecules but a lower clearance of small molecules. Through predilution HF, with on-line ultrafiltration, the clearance of small solutes increased substantially. In HDF, convection is combined with

diffusion, and with this mechanism the clearance of small-, middle- and large-sized molecules can be achieved to more or less the same extent (Ledebro 1998). Both HF and HDF are referred to as convective therapies, although the latter also implies a diffusive mechanism. For both treatments, high-flux dialysis membranes are used, which are also used for so-called high-flux HD, without the use of a convective mechanism. With a combination of convection and diffusion, there is a more efficacious clearance of solutes than with diffusion alone, since molecules that are not removed by simple diffusion can be removed by convection (Ledebro 1998).

Initiation of maintenance HD treatment, although with high-flux polysulfone membranes, has been shown to be ineffective in reducing the serum levels of important inflammatory markers (Pupim, Himmelfarb et al. 2004). A number of previous studies have suggested that the type of dialysis membrane (low-flux or high-flux) is associated with differences in long-term outcome of patients undergoing HD, both in terms of morbidity and mortality (Chauveau, Nguyen et al. 2005; Hornberger, Chernew et al. 1992; Woods and Nandakumar 2000).

The HEMO study, which is the first large randomized clinical trial on patient outcome depending on membrane permeability, failed to show any difference in all-cause mortality between high-flux and low-flux HD, except for some subgroups of patients (Eknoyan, Beck et al. 2002; Cheung, Levin et al. 2003; Rocco, Cheung et al. 2005). The HEMO study has been criticized for several flaws, among others the inclusion of both incident and prevalent HD patients and the exclusion of patients with a high comorbidity and severe malnutrition, meaning the study is not totally representative for the HD population, and its results should be considered with discernment (Locatelli 2003). Reports from the HEMO study indicate that middle-sized molecules, e.g. parathyroid hormone, β_2 -microglobulin, advanced glycosylation end products (AGEs), granulocyte inhibitory proteins, advanced lipoxidation end products (ALEs), advanced oxidation protein products (AOPPs) and leptin (Horl 2002) are associated with systemic toxicity and that their accumulation predisposes dialysis patients to severe infections. An increased clearance of these molecules, e.g. β_2 -microglobulin, by high-flux hemodialysis is associated with a lower mortality by infectious disease (Cheung, Greene et al. 2008). An increased removal of middle-

sized molecules could also have positive effects of the cardiovascular system (Vanholder, Argiles et al. 2001; Vanholder, Baurmeister et al. 2008).

The DOPPS study (Dialysis Outcomes and Practice Patterns Study) revealed that patients on high-flux HDF had a 35 % lower mortality rate than patients on low-flux HD (Canaud, Bragg-Gresham et al. 2006; Canaud, Chenine et al. 2008). However, the DOPPS study is not randomized, and therefore there is great concern about possible selection bias.

In a Cochrane database review by Rabindranath et al., the authors were unable to demonstrate a significant advantage with convective therapies over low-flux HD with regard to clinical outcomes such as mortality, dialysis-related hypotension and hospitalization (Rabindranath, Strippoli et al. 2006). The results highlight the need of large randomized trials to evaluate any difference in outcome between high-flux and low-flux therapies.

The MPO-study (Membrane Permeability Outcome) is a European randomized clinical trial on the effect of high-flux treatment in a large HD population. It is a prospective study which analyses the long-term effect of membrane permeability on clinical outcomes such as mortality, morbidity, vascular access survival and nutritional status. The authors did not find any significant survival benefit overall by high-flux hemodialysis vs. low-flux hemodialysis. However, for the dialysis population with low serum albumin and for patients with diabetes mellitus, a significantly lower mortality rate was observed by high-flux hemodialysis compared to low-flux hemodialysis (Locatelli, Martin-Malo et al. 2009).

1.5 GENE TRANSCRIPTION IN CKD

1.5.1 The IL-1 family

The IL-1 family consists of several members, among which IL-1 α , IL-1 β and IL-1Ra (IL-1 receptor antagonist) are the most important. Their functions are displayed in Table 3. IL-1 as a proinflammatory cytokine stands for a great part of the pathologic changes associated with HD (Descamps-Latscha 1993). In HD patients, elevated IL-1Ra levels have been demonstrated as a response to transfer of cytokine-inducing substances (LPS, various other endotoxins and exotoxins) through dialysis membranes (Schindler, Krautzig et al. 1996). In patients with CKD stage 5 and HD patients, there is an impaired endotoxin-induced interleukin-1 β secretion from mononuclear cells, which could be due to an impaired

processing of the IL-1 β precursor protein into the mature molecule by ICE (Lonnemann, Barndt et al. 1995). IL-1 gene cluster polymorphisms have been demonstrated to predict risk for progression of CKD to ESRD (Wetmore, Hung et al. 2005).

Member	Mechanism of action	Function	References
IL-1α	The IL-1 biologically inactive precursor peptide is cleaved and activated by IL-1 converting enzyme (ICE), an intracellular cysteine protease (caspase-1), and various matrix metalloproteinases. Activates the intracellular signaling by MAP kinases and heat shock protein 27 by binding to IL-1 receptor 1.	Increases the expression of adhesion molecules (ICAM-1 and VCAM-1). Promotes endothelial transmigration of inflammatory cells. Increases transcription of proinflammatory cytokines (e.g. TNF, IL-6, IL-12, TGF β , MIP-1 α , MMPs and IL-8) and SOD2. Central function in acute and chronic inflammatory diseases. Mediator of local inflammation.	(Dinarello 2002; Dinarello 1996; Dayer 2002)
IL-1β	Acts synergistically with several proinflammatory cytokines.	Systemic hormone-like soluble mediator.	
IL-1Ra	Antagonist with a high affinity to the IL-1 receptors.	Antagonist of IL-1 functions.	

Table 3. Members of the IL-1 family.

1.5.2 IL-1 receptors

IL-1 receptors 1 and 2 (IL-1R1 and IL-1R2) have affinity for IL-1 α , IL-1 β and IL-1Ra (McMahan, Slack et al. 1991). Of these, IL-Ra has the greatest affinity to IL-1R1, which has a long cytoplasmatic tail and is responsible for the induction of the intracellular response after binding to IL-1 (Dayer 2002). IL-1R2 has a very short intracellular domain and causes no signal transduction in the cell. It has therefore been called a “decoy” receptor. Binding to this receptor inactivates the function of IL-1 (Dinarello 2002).

Binding of IL-1 α to IL-1R1 results in phosphorylation of specific IRAKs and TRAF6. Subsequent phosphorylations lead to the release of NF κ B and transcriptional regulation (Dinarello 2002). Both the cytosolic and nuclear activities of IRAK-1 are involved in NF κ B-dependent transcriptional regulation (Liu, Park et al. 2008). IRAK-1 can also mediate IL-8 gene up-regulation through interaction with neutrophil elastase (Walsh, Greene et al. 2001).

1.5.3 Superoxide dismutase 2 (SOD2)

SOD exists as three different isoenzymes. SOD1 or CuZn-SOD was the first enzyme to be characterized. It is a homodimer containing copper and zinc. It is found almost exclusively in the cytoplasm of cells. SOD2 or MnSOD is a manganese-containing tetramer and is localized exclusively in the mitochondria of aerobic cells. SOD3 or EC-SOD is a tetramer containing copper and zinc localized in extracellular spaces and is tissue specific (Zelko, Mariani et al. 2002). SOD2 converts superoxide anions to hydrogen peroxide (Johnson and Giulivi 2005). SOD2 is expressed in many cell types and is up-regulated by several factors, such as IL-1, IL-4, IL-6, TNF α , LPS, oxidative stress, hypoxia and IFN γ (Elsakka, Webster et al. 2007). After transcription, SOD2 production is regulated by a RNA-binding protein which enhances its translation (Zelko, Mariani et al. 2002). SOD2 plays an important role in respiratory burst and phagocytosis, but also acts as a ROS detoxifier.

1.5.4 Other markers

ENA-78 (epithelial neutrophil-activating protein-78, epithelial cell-derived neutrophil attractant-78, or CXCL5) is expressed by neutrophils and macrophages stimulated with LPS. ENA-78 works as a chemotactic factor for neutrophils (Adams and Lloyd 1997) by binding to CXCR2 receptors with high affinity, promoting migration, degranulation, respiratory burst and expression of activated adhesion molecules (Van Den Steen, Wuyts et al. 2003). ENA-78 has also been correlated with acute coronary syndrome, and is blocked with statin treatment (Zineh, Beitelshees et al. 2008).

CD40 is a member of the TNF-receptor superfamily (TNFSF5). It is generally expressed on B-cells, but can be expressed by neutrophils after stimulation with certain cytokines and LPS (Yamashiro, Kamohara et al. 2001). CD40 activates B-cells, lymphocytes and macrophages.

TGF β (transforming growth factor beta) has several functions such as controlling cellular proliferation and differentiation in many different cell types. TGF β is released to the extracellular matrix in a latent form, and there gets activated through different mediators, e.g. ROS, integrins and matrix metalloproteinases (Annes, Munger et al. 2003; Wipff and Hinz 2008).

IL12A codes for IL-12 α , a proinflammatory cytokine, produced principally by monocytes/macrophages and dendritic cells (D'Andrea, Rengaraju et al. 1992; Macatonia, Hosken et al. 1995) but also neutrophils (Cassatella, Meda et al. 1995). IL12A activates lymphocytes and natural killer T cells (NK cells) (Kobayashi, Fitz et al. 1989). LPS is a potent inducer of IL12A, after interaction with CD14 on the cell surface (D'Andrea, Rengaraju et al. 1992).

1.6 THE HL60 CELLS

HL60 is a leukemic promyelocytic cell line, first described by Collins et al. (Collins, Ruscetti et al. 1978). The cell line has some unique features, such as being a growth-factor-independent immortal cell line with distinct myeloid characteristics (Birmie 1988). HL60 cells grow in suspension culture with a doubling time that can vary from 20 hours to 45 hours depending on the subtype of cell line. The cells display a typical myeloblastic/promyelocytic morphology. After differentiation during incubation with DMSO or retinoic acid (Collins 1987), the cells tend to a progressive decrease in cell size, condensation of nuclear material and organization of the nuclei into the typical characteristic of banded and segmented nuclei in mature neutrophils (Collins 1987). HL60 cells can also differentiate into monocytes/macrophages by incubation with 1, 25-dihydroxy-vitaminD₃, phorbol esters and sodium butyrate (Birmie 1988).

1.7 OXIDATIVE STRESS AND ENDOTHELIAL DYSFUNCTION

The incidence and prevalence of cardiovascular disease is highly elevated in CKD patients (Levey and Eknoyan 1999; Schillaci, Reboldi et al. 2001; Zoccali 2002; Collins 2003; Vanholder, Massy et al. 2005).

The heart and kidney functions are interconnected by various factors that act in a synergistic way to maintain fluid levels, blood pressure and electrolyte homeostasis. The combination of heart and kidney failure has been named the cardiorenal syndrome, a condition with high hospitalization and mortality rates (Bongartz, Cramer et al. 2005; Makaritsis, Liakopoulos et al. 2006). This has been considered a result of an inflammatory state (Zimmermann, Herrlinger et al. 1999) and oxidative stress on the endothelium (Cachofeiro, Goicochea et al. 2008). Neutrophils from patients with CKD are proposed to be in a primed state (Ward and McLeish 1995; Sela, Shurtz-Swirski et al. 2005), which affects important

cellular functions as well as the state of oxidative stress. Several markers for oxidative stress and endothelial dysfunction have been identified. There is a clear connection between the increase in acute phase proteins and proinflammatory cytokines and endothelial dysfunction (Bolton, Downs et al. 2001; Kaysen and Kumar 2003).

The oxidative stress in patients on HD is multifactorial. There is an imbalance between the generation of ROS and the antioxidative system. HD with low-flux bioincompatible membranes activates NADPH oxidase, which enhances oxidative stress (Morena, Cristol et al. 2002; Samouilidou, Grapsa et al. 2007). The NADPH complex is also up-regulated by LPS fragments in the dialysate, activating neutrophils and monocytes. Oxidation of LDL is an important step in the process of early arteriosclerosis. During inflammation, the anti-oxidative action of HDL is suppressed, leading to higher amounts of oxidized LDL (Maggi, Bellazzi et al. 1994; Artl, Marsche et al. 2000).

2 AIMS

The objective was to study leukocyte function and inflammatory markers in CKD patients and patients on high-flux HD/HDF.

The specific aims were:

- To study the up-regulation of CD11b/CD18, production of hydrogen peroxide and apoptosis of *in vivo* extravasated monocytes and neutrophils at the site of an induced interstitial inflammation in patients on high-flux HD/HDF compared to healthy subjects **(I)**.
- To study the chemotactic activity in terms of chemokine production at the site of interstitial inflammation in patients on high-flux HD/HDF, compared with healthy subjects, as a potential mechanism behind the differences observed in leukocyte function between patients on high-flux HD/HDF and patients on low-flux bioincompatible HD **(II)**.
- To compare the *in vitro* model by LPS stimulation with the *in vivo* extravasation model for studying neutrophil mobilization of CD11b/CD18 and transcriptional activation **(III)**.
- To study the differences in gene transcription of important inflammatory mediators in neutrophils from CKD patients and healthy subjects, following *in vitro* stimulation with LPS, and the consequence of an impaired SOD2 transcription in terms of neutrophil respiratory burst and production of proinflammatory mediators **(IV)**.

3 METHODS

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

3.1 PATIENT CHARACTERISTICS

Patients with CKD and patients on high-flux HD or HDF were recruited from the Department of Nephrology at the Karolinska University Hospital, Stockholm, Sweden. Informed consent was obtained from all participants and the studies were approved by the Ethics Committee of Karolinska University Hospital and the Regional Ethics Committee of Stockholm, Sweden. None of the patients or healthy controls had any signs of infection or was on any anti-inflammatory medication at the time of the study.

Study I and II: 10 patients (7 males and 3 females) with a mean age of 64 years (range 33-74 years), on on-line HDF or high-flux HD. All patients were dialyzed with high-flux polysulfone membranes. Mean dialysis time was 14 ± 1 hour/week and mean Kt/V was 1.7 ± 0.3 . For the causes of ESRD and further patient characteristics, please view Table 4. Eleven healthy subjects (3 males and 8 females) with a mean age of 54 years (range 32-75 years) were examined as a control group.

Study 3: For the study of *in vivo* extravasated neutrophils, we recruited 10 patients (7 males and 3 females) with CKD stage 3-4, mean age 55 ± 7 years with a mean GFR of 45 ± 23 ml/min x 1.73 m². The causes of CKD were glomerulonephritis (4 patients), nephrosclerosis (4 patients) and polycystic kidney disease (2 patients). Eleven age-matched healthy subjects (5 males and 6 females), mean age 55 ± 15 years, were used as a control group.

Neutrophils for RNA purification were also isolated from blister exudates from 3 patients with CKD stage 4-5 and 3 healthy subjects, after *in vivo* extravasation by the skin chamber technique.

Study 3 and 4: Thirty patients with CKD stages 3-5 (18 males and 12 females) with a mean age of 52 ± 11 years and a mean GFR of 34 ± 20 ml/min x 1.73 m² measured with iohexol clearance and 31 ± 19 ml/min x 1.73 m² according to the MDRD equation. The causes of CKD were glomerulonephritis (18 patients), polycystic kidney disease (8 patients) and nephrosclerosis (4 patients). Fifteen

healthy subjects were used as a control group (6 males and 9 females), with a mean age of 50 ± 12 years.

Pat no	Access	Age	Type	Cause of renal disease	Comorbidity
1	AV-graft	33	HDF Predilution 30 L	Interstitial nephritis	-
2	AV-fistula	62	HD	Chronic glomerulonephritis	Hypertension, single kidney after tumor surgery
3	AV-fistula	69	HDF Postdilution 20 L	Chronic glomerulonephritis	Hypertension, cerebrovascular disease, left ventricular hypertrophy
4	AV-fistula	71	HDF Postdilution 25 L	Polycystic kidney disease	Hypertension, left ventricular dysfunction, atrial flutter
5	AV-fistula	68	HDF Postdilution 24L	Chronic glomerulonephritis	Hypertension, left ventricular dysfunction, myocardial infarction, ischaemic heart disease
6	AV-fistula	72	HDF Predilution 60 L	Nephrosclerosis	Diabetes mellitus, hypertension, atrial flutter
7	AV-fistula	64	HDF Postdilution 20 L	Interstitial nephritis	Atrial flutter, pacemaker, recurrent urinary tract infections, ischaemic heart disease, left ventricular hypertrophy and dysfunction
8	AV-fistula	74	HDF Postdilution 24 L	Nephrosclerosis	Hypertension, ischaemic heart disease
9	AV-fistula	60	HD	Chronic glomerulonephritis	Hypertension, ischaemic heart disease, myocardial infarction
10	CDC	63	HDF Postdilution 14 L	Nephrosclerosis	Hypertension, single kidney after tumor surgery

Table 4. Patient characteristics for Study I and II. AV = arterovenous. CDC = central dialysis catheter.

3.2 THE SKIN CHAMBER METHOD FOR *IN VIVO* EXTRAVASATION

The skin chamber technique is well documented and has been used by a number of investigators to study transmigration and recruitment of leukocytes (Scheja and Forsgren 1985; Follin 1999; Thylen, Lundahl et al. 2000; Jacobson, Thylen et al. 2002; Theilgaard-Monch, Knudsen et al. 2004; Dadfar, Jacobson et al. 2007; Paulsson, Dadfar et al. 2007).

We used the skin chamber technique to induce a site of interstitial inflammation on the volar surface of the forearm, by applying a constant vacuum of 300 mm Hg

and gentle heating at 39 °C for 2-3 hours. On the following morning, 12-14 hours after the blister formation, the blister fluid was aspirated, pooled and placed on ice for further analysis and the blister areas were washed with PBS, which was then added to the collected samples.

The roofs of the blisters were then carefully removed and a sterilized open-bottom plastic skin chamber was mounted over each of the exposed blister floors. The chambers were filled with 1 ml of autologous serum or PBS, both containing heparin. Administration of serum or PBS was done in order to induce a site of intense inflammation and a site of intermediate inflammation. After a total of 10 hours of incubation, the blister fluid in the respective skin chamber was aspirated and placed on ice. During the 10 hours incubation, patients went through high-flux HD/HDF (study I and II). The skin chambers were washed with equal volumes of PBS that was added to the collected samples.

The chamber exudates were centrifuged and the cell-free supernatants frozen before they were analyzed. The cell pellets were dissolved in PBS for further experiments.

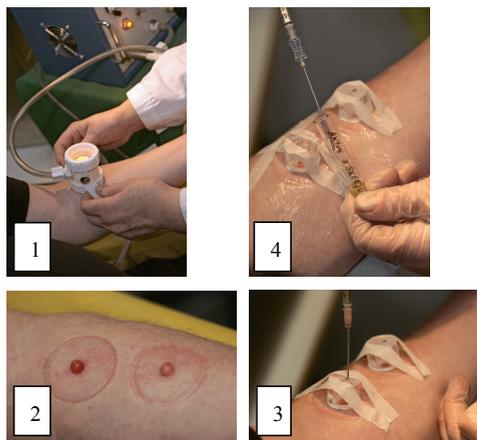


Figure 3. The skin chamber method. 1) Skin blister formation. 2) Skin blister at time 0 hours. 3) Challenge with serum or buffer. 4) Collection of samples after incubation.

3.3 ANALYSES BY FLOW CYTOMETRY

Flow cytometry or FACS (fluorescence-activated cell sorting) is a method by which cells are scanned by a laser. Forward scatter (FSC) measures cell size. Side

scatter (SSC) measures the density of the particle/cell, which depends on the number of granules and membrane size.

In our study, we used Epics Elite (Beckman Coulter, Inc., Hialeah, Fla., USA). This instrument recognizes different leukocyte cell populations by their light-scattering properties. The leukocytes are presented in a two-parameter scatter plot histogram. The FSC signal, representing cell size, is expressed on the y-axis while the SSC signal represents cell granularity and is expressed on the x-axis. Mean fluorescence intensity (MFI) values for the different analyses of cell functions (CD11b/CD18 expression, hydrogen peroxide formation and apoptosis) can also be measured and quantified.

3.3.1 Cell counts

Cell counts in peripheral blood and in blister exudates were made using flow cytometry and the number of neutrophils, monocytes and lymphocytes in the blisters were calculated as a percentage of the total cell population.

3.3.2 CD11b/CD18 expression

The CD11b/CD18 expression on leukocytes both unstimulated and after stimulation with fMLP and PMA, was studied through immunostaining with phycoerythrin-conjugated monoclonal mouse anti-human CD11b/CD18 receptor. Using flow cytometry, the density of the adhesion molecule expression on neutrophils and monocytes was presented as mean fluorescence intensity (MFI) of the gated leukocyte population within a chosen field.

3.3.3 Respiratory burst

Analysis of leukocyte hydrogen peroxide formation, after stimulation with fMLP or PMA, was performed using the 2', 7'-dichlorofluorescein diacetate (DCFH-DA) method.

3.3.4 Apoptosis

We stained leukocytes with Annexin V and propidium iodide (PI) to define cells that were in an early or late apoptotic state.

3.4 CHEMOKINES IN BLISTER FLUID AND IN THE PERIPHERAL CIRCULATION

Chemokines were analyzed with commercially available immunoassays (Quantikine®, R&D Systems Inc. Minneapolis, MN, USA). All immunoassays were used in accordance with the manufacturer's instructions.

3.5 NEUTROPHIL ISOLATION AND *IN VITRO* LPS STIMULATION

Neutrophils were separated from mononuclear cells in peripheral blood by density centrifugation through Percoll/Ficoll Paque. Erythrocytes were lysed by addition of cold isotonic lysing solution. The samples were washed and centrifuged, and the remaining neutrophil pellet was resuspended in HEPES-RPMI1640 with 5 % heat-inactivated fetal bovine serum (FBS).

The samples were divided into one sample that remained unstimulated and one that was stimulated by incubation with LPS at 37 °C for four hours. After washing and centrifugation, neutrophil CD11b/CD18 was measured through flow cytometry to verify stimulation by LPS. The pellets were then frozen for further analysis.

3.6 HL60 CELL DIFFERENTIATION AND INHIBITION OF SOD2 AND MEASUREMENT OF RESPIRATORY BURST

HL60 cells were induced to differentiate toward the neutrophil lineage by incubation with DMSO (dimethyl sulphoxide). The cells were then transfected with small interfering RNA (siRNA) against SOD2 through electroporation.

Production of hydrogen peroxide by differentiated HL60 cells, either PMA-stimulated or unstimulated, with or without previous inhibition of SOD2, was measured by flow cytometry.

3.7 RNA EXTRACTION AND QUANTITATIVE PCR

RNA was extracted from neutrophils and HL60 cells. cDNA was synthesized by Superscript II reverse transcriptase and real-time or quantitative PCR reactions for specific mRNAs (SOD2, IL1A, IL-1R1, IL-1R2, IL8RA, TGFβ, CXCL5, IL12A, CD40, IRAK-1 and IL8) were performed. Analysis of the data was performed using Ingenuity Pathway Analysis.

4 STATISTICAL ANALYSIS

Results are expressed as mean \pm SD for the normally distributed data and as median and range or interquartile range for the non-parametric data. Statistical significance was determined at a p-level of <0.05 .

4.1 PAPER [I-II]

Statistical analysis was performed using ANOVA for repeated measurements and the post hoc Scheffé test for the CD11b/CD18 analyses. Mann-Whitney U test for two independent samples or the Wilcoxon matched pairs test for two dependent samples was used to analyze data for the hydrogen peroxide formation, apoptosis and chemokine concentrations. Regression analysis was performed to study the correlation between the level of chemokines and the total count and CD11b/CD18 expression of extravasated neutrophils and monocytes.

4.2 PAPER [III-IV]

The Mann-Whitney U test was used to determine differences in neutrophil CD11b/CD18 expression, neutrophil count and gene transcription fold change (where appropriate). Student's t-test was used for statistical analysis between populations of differentiated HL60 cells (SOD2 inhibited/uninhibited, before and after PMA stimulation) regarding hydrogen peroxide production.

5 RESULTS AND DISCUSSION

5.1 CD11b/CD18 EXPRESSION ON LEUKOCYTES

There was a similar expression of CD11b/CD18 on monocytes and neutrophils in patients on high-flux HD/HDF and healthy subjects in the peripheral circulation and at the three sites of interstitial inflammation. *In vitro* activation with fMLP induced a significant increase in the expression of CD11b/CD18 on monocytes and neutrophils in the peripheral circulation and at the sites of interstitial inflammation, both in patients on high-flux HD/HDF and healthy subjects.

There was no significant difference in the total number of leukocytes between patients on high-flux HD/HDF and healthy subjects at the inflammatory sites.

In our study of leukocytes from patients on high-flux HD/HDF, leukocytes were studied at their actual site of action, namely after *in vivo* extravasation. This is advantageous, since leukocyte function in patients with CKD or on dialysis has previously almost exclusively been studied on cells collected from the peripheral circulation.

Our findings of a preserved capacity of both monocytes and neutrophils to express CD11b/CD18 at the sites of interstitial inflammation in patients on high-flux HD/HDF may have important biological consequences in terms of preserved performance of leukocyte functions in which the CD11b/CD18 molecule plays a key role (Thylen, Fernvik et al. 1997; Moshfegh, Jacobson et al. 2002). Extravasated neutrophils and monocytes from patients on high-flux HD/HDF showed a maintained response to fMLP as a second inflammatory stimulus after extravasation.

The mechanism behind this preserved leukocyte function in patients on high-flux biocompatible HD/HDF could be the removal of small and middle-sized leukocyte inhibitory molecules by high-flux HD/HDF (Vanholder, De Smet et al. 1994), but the results could also stand for membrane compatibility.

5.2 RESPIRATORY BURST AND APOPTOSIS

Results for respiratory burst (hydrogen peroxide production) in neutrophils and monocytes are displayed in Figures 4-7. The findings indicate the presence of a dose-response phenomenon in terms of leukocyte function at the site of interstitial

inflammation in patients on high-flux HD/HDF, which could be due to leukocyte refractoriness when encountered with a strong inflammatory stimulus. Refractoriness of leukocytes could be caused by previous priming, creating an impaired response to a second activating stimulus.

In both the neutrophil and monocyte populations, we observed no significant differences in the percentage of apoptotic cells (Annexin V+ and Annexin V+ PI+) in the peripheral circulation or at the sites of interstitial inflammation between patients on high-flux HD/HDF and healthy subjects. Clearance of leukocytes via apoptosis from the site of infection is crucial for the coordinated resolution of inflammation. The balance between pro-apoptotic and apoptosis-inhibiting factors is necessary for the maintenance of an effective immune response without the harmful side effects of an excessive neutrophil activation. Previous studies have indicated that CKD is a state that induces apoptosis, but that this is normalized with continuous and high-flux blood purification modalities (D'Intini, Bordoni et al. 2004; Bordoni, Piroddi et al. 2006). This is in accordance with studies showing that dialysis membrane characteristics affect leukocyte cell apoptosis (Martin-Malo, Carracedo et al. 2000; Sardenberg, Suassuna et al. 2006).

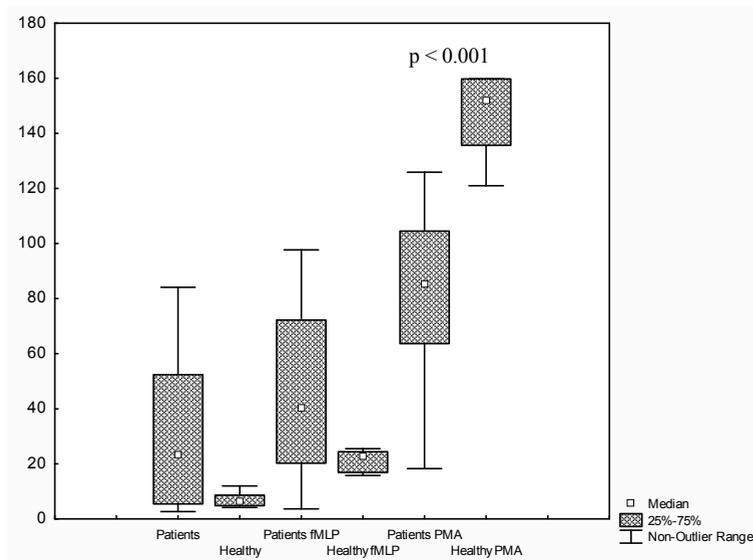


Figure 4. Respiratory burst in neutrophils at the site of an intermediate interstitial inflammation expressed as mean fluorescence intensity (MFI). P is indicated where a significant difference is present.

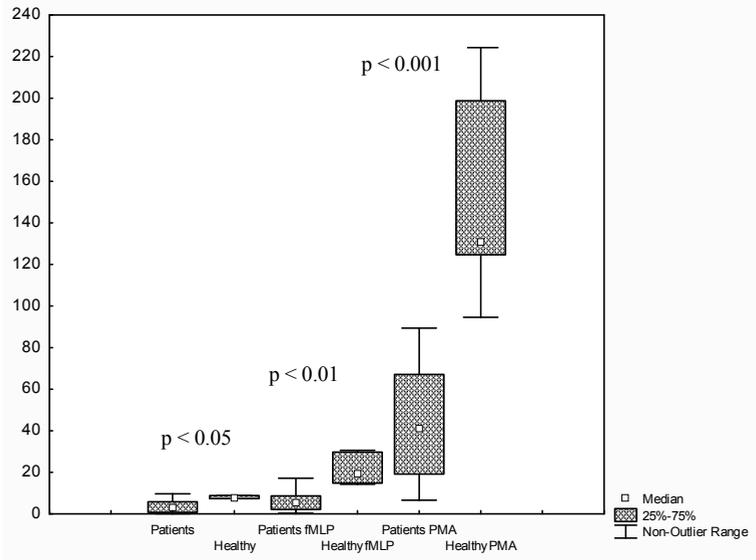


Figure 5. Respiratory burst (MFI) in neutrophils at the site of intense interstitial inflammation.

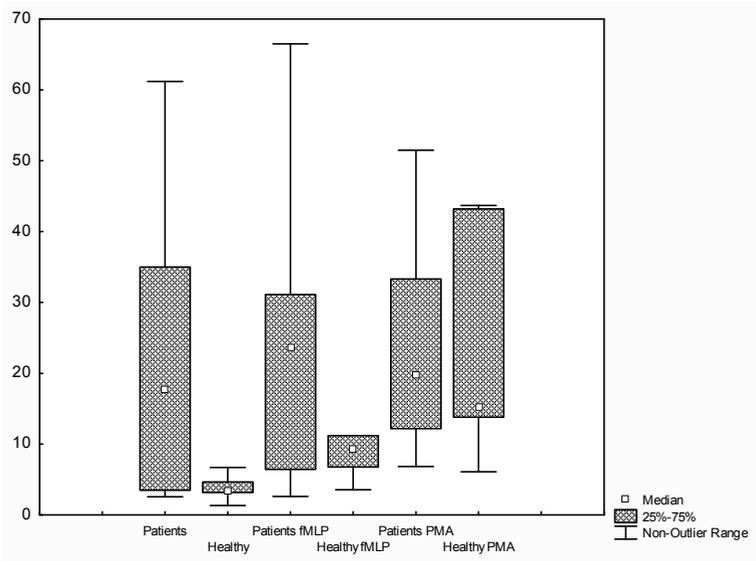


Figure 6. Respiratory burst (MFI) in monocytes at the site of intermediate interstitial inflammation. NS for all comparisons.

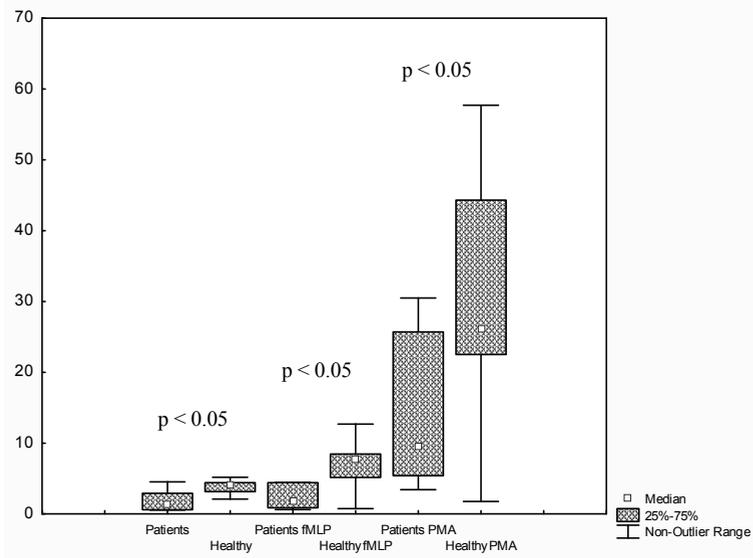


Figure 7. Respiratory burst (MFI) in monocytes at the site of intense interstitial inflammation.

5.3 CHEMOKINES

Patients on high-flux HD/HDF had significantly higher concentrations of MCP-1, MIP-1 α , IL-6, IL-8, TNF α and high-sensitivity CRP (hsCRP) in the peripheral circulation, prior to dialysis treatment, compared with healthy subjects. MMP-9/NGAL serum concentration was similar in patients on high-flux HD/HDF and healthy subjects. Results for MCP-1, MIP-1 α , IL-8 and MMP-9/NGAL concentrations at the sites of interstitial inflammation are published in paper II. Results that are not published in paper II are demonstrated in Table 5.

Significantly higher serum levels of β 2-microglobulin and serum amyloid A (SAA) were observed in patients on high-flux HD/HDF compared with healthy subjects. The serum concentrations of chemokines, hsCRP, SAA and oxidized LDL were not influenced by the high-flux HD/HDF session, while the concentration of β 2-microglobulin was significantly reduced.

The concentrations of MIP-1 α , MMP-9/NGAL and IL-8 were similar in patients and healthy subjects at the sites of intermediate and intense inflammation, and the concentration of MCP-1 was significantly higher in patients on high-flux HD/HDF compared with healthy subjects at the sites of intermediate and intense inflammation. At the site of intermediate inflammation, the concentration of IL-6

and TNF α was significantly higher in patients compared with healthy subjects, reflecting a high inflammatory activity (Table 5).

There were no significant correlations between the concentrations of chemokines or the gradient between the concentration in the peripheral circulation and the interstitium, and the recruitment of neutrophils and monocytes and their expression of CD11b/CD18 at the site of interstitial inflammation.

In the present study we demonstrate that the interstitial concentrations of determinants for monocyte and neutrophil recruitment and CD11b/CD18 expression in patients on high-flux HD/HDF equal or are higher than the corresponding concentrations in healthy individuals.

The higher concentration of MCP-1 and equal concentration of IL-8, MMP-9/NGAL and MIP-1 α at the sites of intermediate and intense inflammation in patients on high-flux HD/HDF could be of importance for the maintained capacity of leukocytes to extravasate and mobilize CD11b/CD18 in response to both an intermediate and an intense inflammatory stimulus, compared with healthy subjects (Paper I). These data contrast with our previous studies on patients with CKD or patients on peritoneal dialysis, in which the concentrations of MCP-1 and IL-8 are significantly lower, coupled with an impaired capacity to up-regulate CD11b/CD18 on neutrophils at sites of interstitial inflammation (Dadfar, Lundahl et al. 2004; Dadfar, Lundahl et al. 2004; Dadfar, Lundahl et al. 2004). The results of our study support a preserved neutrophil and monocyte function in terms of extravasation and activation at the inflammatory focus.

	IL-6 (pg/mL)	TNF α (pg/mL)	Ox LDL (U/L)	β -microglobulin (mg/L)	SAA (mg/L)
Serum 0 h					
Patients	7.8 (5.2-15.4)	3.8 (3.5-4.8)	67.4 (51.3-75.8)	25.5 (22.0-40.0)	13.1 (6.5-27.8)
Healthy	1.8 (0.9-3.5)	0.73 (0.59-1.2)	78.0 (54.2-93.4)	1.5 (1.4-1.7)	2.8 (1.4-4.7)
	p < 0.01	p < 0.001	NS	p < 0.001	p < 0.01
Serum 10 h					
Patients	8.3 (7.0-20.5)	3.1 (2.9-3.6)	65.9 (58.0-94.2)	15.5 (13-17)	13.8 (4.0-24.0)
Healthy	-	-	-	1.5 (1.3-1.7)	3.4 (1.5-5.1)
				p < 0.001	p < 0.05
Intermediate inflammation					
Patients	13,458 (9,857-19,344)	359 (174-1,430)	-		
Healthy	3,639 (1,329-7,066)	153 (78-178)	-		
	p < 0.001	p < 0.05			
Intense inflammation					
Patients	7,937 (6,835-18,250)	397 (191-1,463)	-		
Healthy	4,346 (2,805-7,210)	396 (305-702)	-		
	p < 0.05	NS			

Table 5. Concentrations of inflammatory markers in the peripheral circulation and at the sites of interstitial inflammation in patients on high-flux HD/HDF and healthy subjects, expressed as median and interquartile range.

5.4 IN VITRO LPS-ACTIVATED NEUTROPHILS

After stimulation with LPS, there was a highly significant up-regulation of CD11b/CD18 on the cell surface of neutrophils from patients with CKD and healthy subjects ($p < 0.001$ for both comparisons), which indicates that both cells from healthy subjects and patients with CKD were activated by the LPS stimulation. The relationships between the different gene products that we studied are illustrated by the ingenuity pathway map in Figure 8. As a response to LPS stimulation, neutrophils from patients with CKD display either a very weak up-regulation (IL1R2) or even a down-regulation (SOD2, IL1R1 and IL1A), expressed as fold change of gene transcription. Neutrophils from healthy subjects respond with an up-regulation of SOD2, IL1A, IL1R1 and IL1R2 to stimulation

with LPS. Neutrophils from both patients and healthy subjects respond with an up-regulation of IL8RA (IL-8 receptor α) to LPS stimulation.

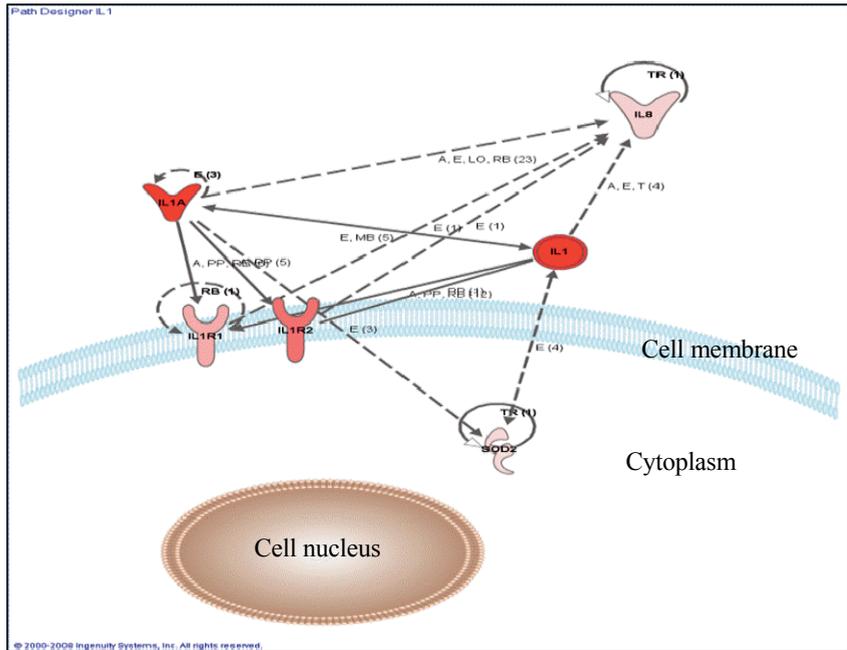


Figure 8. Relationship between the gene products analyzed. The intensity of the red color indicates the difference in up-regulation between patients with CKD and healthy subjects. Arrows indicate indirect (---) and direct (—) activation.

5.5 INHIBITION OF SOD2 AND RESPIRATORY BURST

To explore the physiological consequence of down-regulated SOD2, we inhibited SOD2 with siRNA in differentiated HL60 cells. We observed a significantly lower transcription of IL1A, IL1R1, IL1R2 and IL8RA after stimulation with PMA in cells with inhibited SOD2 *de novo* production. These data indicate a central role of SOD2 in the up-regulation of important proinflammatory mediators in neutrophils. PMA stimulation of differentiated HL60 cells with maintained SOD2 production leads to up-regulation of IL1R1, IL1A and IL8RA and down-regulation of IL1R2 (the IL-1 *decoy* receptor).

We found a correlation between PMA-stimulated differentiated HL60 cells and LPS-stimulated neutrophils with respect to gene expression changes, supporting

the theory that differentiated HL60 cells may be a suitable experimental model to study neutrophil functions.

Since hydrogen peroxide production, or respiratory burst, is an essential mechanism for neutrophil function, we examined the impact of SOD2 inhibition in differentiated HL60 cells. We found a significant increase in respiratory burst after stimulation with PMA ($p < 0.01$) in both siRNA-treated and non-siRNA-treated cells. However, there was a significantly higher respiratory burst response after PMA stimulation of neutrophils with normal SOD2 *de novo* production than in SOD2 inhibited neutrophils.

In summary, we demonstrate that neutrophils from CKD patients down-regulate SOD2 gene expression after LPS stimulation. Since SOD2 is of central importance for respiratory burst, this finding could help us to further understand neutrophil dysfunction and the increased susceptibility to infections in CKD patients.

SOD2 inhibition by siRNA also had a negative effect on the IL8RA transcription as a response to LPS stimulation. Thus, SOD2 might be involved in the regulation of this central neutrophil chemokine receptor.

We analyzed (unpublished data) the plasma concentration of endothelin-1 as well as important neutrophil and monocyte chemokines and activators (IL-8, MMP-9/NGAL and MIP1 α , MCP-1) in CKD, and observed a significantly higher plasma concentration of endothelin-1, MCP-1 and MIP-1 α ($p < 0.0001$ for all comparisons) in CKD patients compared with healthy subjects, while plasma concentrations of MMP-9/NGAL and IL-8 were similar in CKD patients and healthy subjects. There are reports of higher plasma concentrations of endothelin-1 in patients with CKD compared with healthy subjects (Kohan), and this is consistent with our findings of a higher endothelin-1 plasma concentration in CKD patients. The LPS-induced expression of CXCL5 (ENA-78) measured as fold change in gene transcription was significantly higher in patients with CKD ($p < 0.05$) than in healthy subjects (unpublished data), which points to an activation of circulating neutrophils in CKD patients.

5.6 IN VIVO EXTRAVASATION VERSUS IN VITRO LPS STIMULATION REGARDING CD11b/CD18 UP-REGULATION AND GENE TRANSCRIPTION

After both *in vivo* extravasation and *in vitro* LPS stimulation, there was a significant up-regulation of CD11b/CD18 on neutrophils ($p < 0.001$) both in CKD patients and healthy subjects. Neutrophil CD11b/CD18 was up-regulated 5-6 times both in patients and healthy subjects by the LPS stimulation, which was in the same range as *in vivo* extravasated neutrophils from CKD patients and healthy subjects. However, the LPS stimulation caused a higher mobilization of CD11b compared with *in vivo* extravasation ($p < 0.05$ for CKD patients and $p < 0.001$ for healthy subjects). In neutrophils from patients with CKD and healthy subjects, the fold change in gene transcription of IRAK-1, TGF β and CD40 were similar after *in vitro* LPS stimulation and *in vivo* extravasation, but the fold change in gene transcription of IL8 was higher after *in vivo* extravasation than after *in vitro* LPS stimulation in both CKD patients and healthy subjects. This phenomenon is in line with the physiological mechanisms, because the extravasation process is a strong up-regulator of IL8 at the initial phase of transmigration and the inflammatory response.

CKD patients up-regulate CD40 as a response to both *in vitro* LPS stimulation and *in vivo* extravasation, whereas healthy subjects down-regulate CD40 with both corresponding stimuli. IL12A was up-regulated in CKD patients by both *in vivo* extravasation and *in vitro* LPS stimulation, but in healthy subjects the extravasation model gave a down-regulation of IL12A, while *in vitro* LPS stimulation gave a transcriptional up-regulation of the same gene. This phenomenon of discrepancy between neutrophils from healthy subjects and neutrophils from CKD patients could be due to priming of neutrophils in patients with CKD, leading to CD40 and IL12A up-regulation by both extravasation and LPS stimulation.

The results for CD11b/CD18 mobilization, as well as the similar up-regulation of IRAK-1, TGF β and CD40, suggests the utility of *in vitro* LPS stimulation as an experimental model for studying *in vivo* activated neutrophils.

6 CONCLUSIONS

- I. *In vivo* extravasated monocytes and neutrophils from patients on high-flux HD/HDF have a preserved capacity to mobilize CD11b/CD18, compared with corresponding cells from healthy subjects. Furthermore, monocytes and neutrophils were able to respond to a second signal (fMLP) at the site of interstitial inflammation, indicating an adequate response to bacterial peptides. After the most potent stimulation, both monocytes and neutrophils that had extravasated *in vivo* and been recruited to the site of intense inflammation showed a lower capacity to produce hydrogen peroxide in response to activation compared with corresponding cells from healthy individuals. The apoptotic rate of neutrophils and monocytes were similar in patients and in healthy subjects.
- II. One possible explanation for the preserved ability of monocytes and neutrophils to express CD11b/CD18 in response to an interstitial inflammation in patients on high-flux HD/HDF may be due to the fact that the cells extravasate into a milieu which contains equal or higher concentrations of factors involved in transmigration and CD11b/CD18 expression compared with healthy subjects (MCP-1, IL-8, MIP-1 α and MMP-9/NGAL). The maintained capacity to produce chemokines in the interstitium in patients on high-flux HD/HDF may be due to an increased intradialytic removal of substances that inhibit leukocyte function.
- III. The use of *in vitro* LPS stimulation is demonstrated to be a potential model for *in vivo* activated neutrophils, to study transcriptional activity of proinflammatory mediators and phenotype in terms of CD11b/CD18 expression.
- IV. Neutrophils from patients with CKD have an impaired capacity to produce hydrogen peroxide as a response to invading bacteria. A lower transcription of neutrophil SOD2 in patients with CKD could be one important factor for the impaired production of hydrogen peroxide and transcription of proinflammatory mediators as a response to LPS stimulation.

7 FUTURE PERSPECTIVES

This thesis provides deeper knowledge of the mechanisms behind leukocyte dysfunction in CKD and in patients on renal replacement therapy. Both the innate immune response and the adaptive immune response are complex processes depending on antigen recognition and the action of immune cells to defend the organism against invading bacteria. Patients with CKD and patients on dialysis are at special risk for infections, and this is an important field for further investigation, to minimize the negative acute and long-term consequences of a life-supporting treatment.

SOD2 has a central role in the activation of neutrophil transcriptional machinery and in the production of hydrogen peroxide, which is a central step in phagocytosis and killing of bacteria. It is important to continue analyzing the specific mechanisms of neutrophil function at the site of inflammation in patients with CKD, focusing on uremic toxins and their effects on circulating neutrophils. Furthermore, studies on the precise operating mechanisms responsible for the refractoriness of primed neutrophils when they encounter an inflammatory stimulus are important, since this reaction probably plays an important role in neutrophil dysfunction in CKD patients.

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