SOCS and inflammation in chronic renal failure

M. Mehdi Rastmanesh

SOCS and inflammation in chronic renal failure
Rastmanesh, Mohammad Mehdi

Thesis, University of Utrecht, with a summary in Dutch

ISBN: 978-90-393-4892-5

Printed by: Gildeprint drukkerijen B.V., Enschede

Cover design: M.M. Rastmanesh

SOCS and inflammation in chronic renal failure

SOCS en inflammatie in chronische nierfalen
(met samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. J.C. Stoof, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op donderdag 18 december 2008 des ochtends te 10.30 uur

door

Mohammad Mehdi Rastmanesh

geboren in Shiraz/Iran

Promotor:
Prof.dr. G. Pasterkamp

Co-promotoren:
Dr. B. Braam
Dr. H.A.R. Bluyssen

The studies in this thesis were supported by the Dutch Kidney Foundation (Grant: NSN C03-2062) and Genzyme (GRIP)

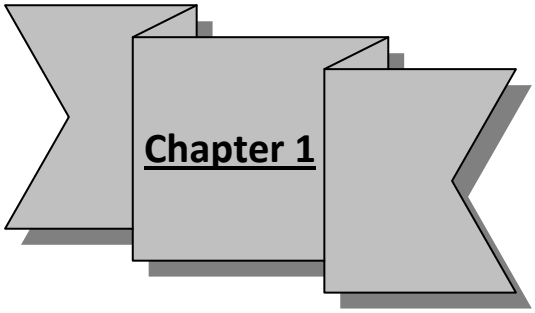
Financial support by the Dutch Kidney Foundation and the Netherlands Heart Foundation for the publication of this thesis is gratefully acknowledged

For my great family

Table of contents

Abbreviations

CIS	Cytokine inducible SH-2 domain containing protein
CKD	Chronic Kidney Disease
CRF	Chronic Renal Failure
CVD	Cardiovascular disease
EC	Endothelial cell
Epo	Erythropoietin
ESRD	End Stage Renal Disease
GFR	Glomerular Filtration Rate
GH	Growth Hormone
IFN	Interferon
IL	Interleukin
Jak	Janus Kinase
MCP-1	Monocyte Chemotactic Protein-1
MNC	Mononuclear cell
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
RT	Reverse transcriptase
SOCS	Suppressors of Cytokine Signaling
STAT	Signal Transducer and Activator of Transcription
TNF	Tumor necrosis factor



General introduction and outline of thesis

Introduction

The prevalence of atherosclerotic cardiovascular diseases (CVD) in patients with chronic renal failure (CRF) is extremely high and accounts for much of the morbidity and mortality in this group (1). In end stage renal disease (ESRD) patients 60% of mortality is caused by atherosclerosis (2). Somehow, a decrease in renal clearance and neurohumoral consequences of renal disease aggravate the atherosclerotic process (3-6). Indeed activation and dysfunction of endothelial cells, increased adhesion molecules (7) and monocyte activation (8) have been demonstrated in renal patients (9, 10) and might be responsible for accelerated atherosclerosis in CRF.

Inflammation, a common feature of CRF, could contribute to the accelerated atherosclerosis (1). In fact inflammation has been shown to be involved in all stages of atherosclerosis (11). Plasma levels of some inflammatory factors (e.g. interleukin-6 (IL-6) and C-Reactive Protein (CRP)) can be viewed as atherosclerosis markers (1). Indeed, recent evidences demonstrate strong association between inflammation and endothelial dysfunction as well as mononuclear cell activation in patients with renal failure (1, 8). A growing body of evidence indicates that management of inflammation could substantially decrease prevalence of CVD (11, 12), pointing to the importance of molecules that are able to diminish or inhibit inflammation. As there is as yet no recognized or even proposed, treatment for renal patients with chronic inflammation (1), it is of paramount importance to find new ways to manage inflammation in patients with renal failure. In this regard understanding how inflammation works at the cellular level may provide new possibilities to control inflammation and consequently atherosclerotic CVD in renal patients.

Atherosclerosis development

Atherosclerosis is initiated when low density lipoproteins accumulate in the intima and activate the endothelium, an early stage of atherosclerosis. Activation of endothelial cells induces expression of adhesion and chemokine molecules that result in recruitment and translocation of monocytes and lymphocytes from the circulation into the intima. This is followed by monocyte differentiation into macrophages and later on to foam cells along with increased numbers and activation of T-helper-1 (Th-1) cells and proliferation of smooth

muscle cells within the lesions. In concert all cells within the lesions secrete pro-inflammatory cytokines that contribute to local inflammation and plaque growth (11). This brief description indicates the central role of endothelial dysfunction and mononuclear cell adhesion and transmigration in initiation and progression of atherosclerosis. Increased systemic inflammation in CRF potentiates endothelial dysfunction and mononuclear cells activation, which could explain facilitated atherosclerosis in CRF.

Chronic renal failure

Chronic renal failure is the clinical syndrome associated with a slow decline in kidney function over time. Chronic renal failure may be caused by a number of disorders, which include long-standing hypertension, diabetes, glomerulonephritis and congenital kidney disease. If renal function declines to a low enough level (GFR below 15 ml/min/1.73 m^2) patients enter the End Stage Renal Disease (ESRD) phase in which renal replacement therapy using dialysis may be necessary. Decreased renal function is accompanied by a dramatic mortality rate in renal patients. The mortality rate among 400,000 American ESRD patients in 2003 was almost 20% yearly (13), which was 40-fold higher than in the general population (14). Strikingly, half of the deaths related to CVD (13, 14). In another report 60% of mortality in ESRD patients was related to atherosclerotic CVD (2). The high prevalence of CVD-related mortality is not only a common problem in ESRD patients, but also in patients with mild-moderate reduced renal function (13), commonly referred to as Chronic Kidney Disease (CKD) patients. The chance of CVD-related death in CKD patients is 5-10 times higher than the chance of entering the ESRD stage (14). The presence of eight million patients with CKD in 2002 in the USA highlights a major global public health concern: the increasing number of renal patients with CVD. Recently, it has been recognized that a decreased level of kidney function per se is an independent risk factor for atherosclerotic CVD (15).

Inflammation & CVD

The underlying cause of increased CVD events in CRF is complex and contains traditional risk factors including hypertension, diabetes, dyslipidemia, and hypertriglyceridemia (16, 17), and non-traditional or uremic-related risk factors including

malnutrition, anemia, oxidative stress, endothelial dysfunction and inflammation (3-6, 17). Inflammation is the focus of this thesis and is not only an independent atherosclerotic CVD risk factor but it also interacts closely with oxidative stress, malnutrition, anemia, and endothelial dysfunction (7, 17, 18). Inflammation in CRF (in 30-50% of patients (1)) is characterized by increased plasma CRP and inflammatory cytokines, in particular IL-6, TNFα, IL-8, IL-10 and IL-1β (1, 7, 19-21).

Available data suggest that pro-inflammatory cytokines play a central role in atherogenesis and mortality in renal patients (1, 7, 8). Importantly, management of inflammation could decrease incidence of CVD events (11, 12), pointing to the importance of molecules that are able to diminish or inhibit inflammation. In this regard understanding how inflammation works at the cellular level may provide new opportunities to control inflammation and consequently atherosclerotic CVD in renal patients.

Peripheral blood mononuclear cells activation in CRF

Mononuclear cells (MNC) are actively involved in atherosclerosis development (11). Renal failure is accompanied by changes in the activation, phenotypes and number of mononuclear cells (8, 22). For instance, the number of activated monocytes is increased (22), even though lymphopenia occurs in CRF (23). Previous reports indicate monocyte defects and a decreased proportion of functional T-cell subsets in renal patients (24-26). Simultaneously, a wide variety of factors in ESRD, are capable of stimulating monocytes/macrophages to release inflammatory cytokines like IL-6, and TNFα, which might be the cause of increased inflammation in CRF and further activation of MNC (27, 28). Furthermore, cell surface adhesion molecules on monocytes are increased in CRF (29) that enhance adhesion of monocytes to the endothelium. Hemodialysis skews T-cell differentiation towards a Th-1 subset capable of producing pro-inflammatory cytokines (e.g. TNFα and IFNγ), which also favors atherogenesis (30-32). Together, these data indicate that mononuclear cells are affected in CRF and they contribute to the production of inflammatory cytokines. *This could link mononuclear cell activation to systemic inflammation in CRF*.

Endothelial cells dysfunction in CRF

The endothelium is a functional barrier between vessel wall and blood stream that fulfills important tasks including coagulation, fibrinolysis, vascular tone, growth, and immune response (33). Endothelial dysfunction is an injury response mechanism, with impaired endothelium-dependent vasodilatation, and increased adhesion of platelets and leukocytes, both critical factors in atherosclerosis development (7, 33). Endothelial dysfunction is common in patients with moderate renal failure (34) and also in ESRD patients undergoing hemodialysis (35) or peritoneal dialysis (36), characterized by impaired endothelium-dependent vasodilation or increased soluble cell adhesion molecules (7). Inflammation has a central role in endothelial dysfunction (33, 37, 38). A comparison between CRF patients and CVD patients with normal renal function has demonstrated that in both groups endothelial dysfunction was related to increased inflammatory markers (IL-6, TNFα, and CRP), which were most elevated in CRF patients (7). Furthermore, in vitro studies indicate that exposure of endothelial cells (EC) to pro-inflammatory cytokines induces production of cell surface adhesion molecules and impairs endothelium-dependent vasodilatation (33). *This could link systemic inflammation (that exists in CRF) to atherosclerotic CVD.*

Cytokine signaling through the Jak/STAT pathway

Many inflammatory cytokines including IL-6, and IL-10 that are involved in inflammation and increased in CRF, relay biological information to a wide variety of target cells by activating the Janus Kinase (Jak) / Signal Transducer and Activator of Transcription (STAT) pathway (39), that mediates the translation of extra-cellular signals to gene transcription.

Briefly, interaction between cytokines and their cognate receptors, brings the receptor-bound Janus kinases (Jak1-3 and Tyk-2) into proximity, resulting in trans-phosphorylation and activation of Jak kinases. In turn activated Jak kinases, phosphorylate specific tyrosine residues within the cytoplasmic region of the receptor. This provides docking sites for STAT molecules (STAT1-4, 5a-b and 6). STAT monomers associate with the phosphorylated tyrosine sites on cytokine receptors and become tyrosine-phosphorylated through the action of Jak kinases. Activated (phosphorylated) STATs are then released from

the receptor. They are now able to form homo- or heterodimers, a necessary step for initiation of transcription. Dimerized STATs translocate to the nucleus and initiate cytokine-specific gene transcription (fig. 1), which can affect vital functions of their target cells, including proliferation, apoptosis, activation, survival, cytokine production and migration (40, 41).

Cytokine signaling is, however, a transient action and it must be tightly controlled to avoid detrimental consequences of excessive stimulation (40, 42). For instance uncontrolled IFNγ signaling causes lymphocyte depletion (43). Therefore, regulatory mechanisms are needed to keep responses to cytokines in check. There are three major classes of negative regulators of the Jak/STAT pathway: 1) protein tryrosine phosphatases (PTPs); 2) protein inhibitors of activated STAT (PIAS); and 3) suppressors of cytokine signaling (SOCS) proteins (40, 42, 44, 45). SOCS proteins provide long-lasting inhibition of the Jak/STAT pathway and are the topic of this thesis.

SOCS expression and mechanism of inhibition of Jak/STAT

Since their discovery, SOCS have also been termed Cytokine-Inducible Src homology-2 (SH2) domain-containing protein (CIS) (46), Jak-Binding protein (JAB) or STAT-induced STAT Inhibitor (SSI) (47). The first studies on SOCS focused on the role of SOCS as inhibitors of Jak/STAT signaling (46, 48). Very soon it became clear that SOCS are important players in many inflammatory diseases (49, 50). Recently, we suggested that SOCS could also be considered as intracellular indicators of inflammation.

a. SOCS expression

The SOCS family comprises eight molecules (SOCS-1-7 and CIS-1). Each SOCS contains a central SH2 domain, an N-terminal domain of variable length and sequence, and a highly conserved C-terminal domain named SOCS box (40, 51). A small Kinase Inhibitory Region (KIR), located near the N-terminus of the SH2-domain, has only been identified in SOCS-1 and SOCS-3. KIR is believed to enhance binding to and inhibition of Jak activity by acting as a pseudo-substrate (41, 52).

In general, SOCS proteins are not present or only expressed at very low levels in non-stimulated cells (40, 53), but SOCS expression is rapidly induced by a broad range of cytokines and serves as a tightly linked negative-feedback mechanism to limit cytokine-induced cellular activation (40). STAT proteins appear to mediate SOCS gene expression, as several SOCS genes have STAT binding sites in their promoters (40). Generally, cytokine stimulation results in up-regulation of several members of SOCS family. In contrast, SOCS-3 is more specifically induced by some cytokines (40). For instance interleukin-10 (IL-10) stimulation of monocytes induces only SOCS-3 expression (54). To date, no information is available regarding whether increased systemic inflammation in CRF affects SOCS expression in MNC and EC.

b. SOCS and inhibition of Jak/STAT

Once expressed, SOCS proteins inhibit responses to cytokines by down-regulating the Jak/STAT pathway. In this context it is important to realize that the inhibitory effects of SOCS are not limited to the cytokines that caused their induction, but also provide a cross-inhibitory mechanism between cytokine pathways. SOCS inhibits Jak/STAT activation using three distinct mechanisms (40, 42); 1) suppress the catalytic activity of Jak Kinases through direct interaction with Jaks via its SH2 domain (e.g. SOCS-1) (52), 2) initial docking onto the cytokine receptor subunit through its SH2 domain, and then interacting with Jak (e.g. SOCS-3) (40), 3) SOCS competes with STAT for binding to phosphotyrosine residues on the receptor (e.g. CIS-1) (55) (Fig. 1). SOCS proteins can also target their substrates for degradation by the proteasomal protein degradation pathway. The SOCS box acts as an adaptor region, coupling the SOCS proteins to elongins B and C, which in turn recruit the protein complex to the proteasome (42, 56). So far, the role of SOCS-1, SOCS-3, and CIS-1 in inhibition of a wide range of cytokines, growth factors and hormones has been documented (40). SOCS-2 also has an inhibitory effect on cytokine signaling, although under certain circumstances it can also enhance cytokine signaling (40, 57). Unfortunately, our present knowledge about SOCS-4 to SOCS-7 is very limited (40, 45).

Thus, cytokines can induce negative feedback to inhibit Jak/STAT activation via SOCS proteins, thereby dampening the response to both harmful and beneficial substances that

depend upon Jak/STAT activation. However, it is not clear whether SOCS is increased in MNC and EC of CRF patients and how this affects pro- and anti-inflammatory signaling pathways.

c.SOCS as an intracellular indicator of inflammation

As mentioned before, SOCS are induced upon cytokine signaling through Jak/STAT pathway. Therefore, increased SOCS expression also reflects the activation of intracellular inflammatory pathways, indicating whether cells experience inflammation and whether a cellular response is in proportion to increased inflammation in the environment. We suggest that SOCS expression could be used as a new intracellular marker related to inflammation and inflammatory-related diseases such as atherosclerosis. By analogy, loss of SOCS2 expression has recently been associated with cell proliferation and tumor growth in breast carcinoma (58). SOCS expression and its relation to increased inflammation and atherosclerotic CVD in CRF patients was not characterized previously.

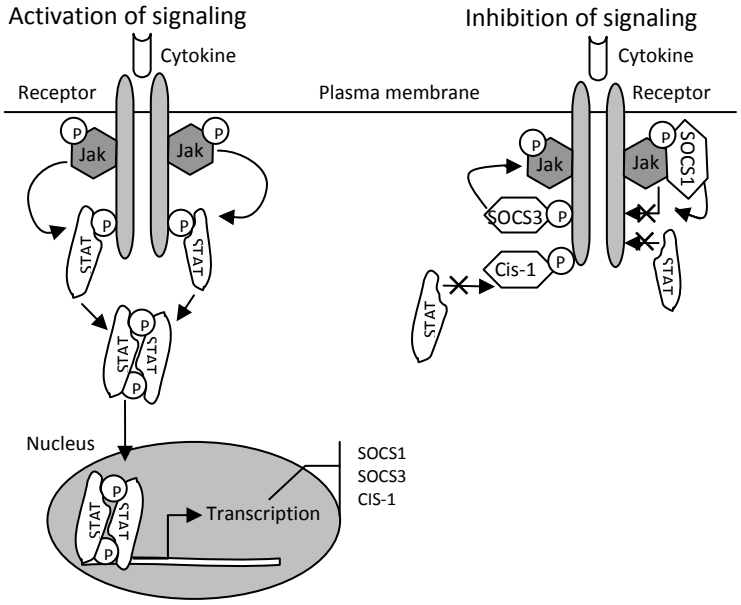


Fig. 1. The molecular mechanism by which SOCS proteins negatively regulate cytokine signaling. It starts with cytokine binding to its receptor and activation of Jak followed by STAT activation, dimerization, translocation to the nucleus and initiation of transcription of genes inculding SOCS. SOCS proteins in turn inhibit Jak/STAT activtion through different mechanisms. SOCS1 binds to the Jaks and inhibits catalytic activity, SOCS3 binds to Jak-proximal sites on the cytokine receptors and inhibits Jak activity, and CIS-1 blocks the binding of STATs to cytokine receptors.

SOCS and inflammatory diseases

SOCS1 and SOCS3 expressions have been demonstrated in different inflammatory diseases (53). SOCS1 expression is increased in lymphocytes and macrophages, as well as keratinocytes and stromal cells that are capable of antigen presentation, but not in granulocytes in Th-1-type inflammatory diseases (e.g. arthritis, colitis and dermatitis) (53). In general, SOCS1 expression inhibits inflammatory diseases (49, 50). In contrast to Th1-type diseases, SOCS3 is increased in Th-2 lymphocytes in Th-2-type diseases (e.g. asthma) and the degree of SOCS3 expression correlates with the severity of the disease (53). Other studies have indicated that dysregulation of SOCS3 is involved in inflammatory diseases in the gastrointestinal tract and joints (59-61). SOCS3 is highly expressed in the colon of mice in an experimental model of colitis, and in intestinal T cells from Crohn's disease patients (63-64). Inhibition of SOCS3 activity, using a dominant-negative transgene, induced hyperactivation of STAT3, and increased the severity of colitis (63). Furthermore, while SOCS3 expression inhibits arthritis (59), it promotes asthma (60), indicating a dual role of SOCS3 in inhibition as well as promotion of inflammatory diseases.

SOCS and CVD

How SOCS can be related to initiation of plaque formation and growth is not well studied. The first step to unravel the role of SOCS in atherogenesis, was taken by Tang and colleagues (61), who indicated strong presence of SOCS-1 and SOCS-3 in atherosclerosis lesions of $ApoE^{-/-}$ mice (61). Interestingly, both SOCS-1 and SOCS-3 co-localized with Mac-2 positive lesion macrophages (61), suggesting the important contribution of macrophages in SOCS induction or reversely the importance of SOCS in macrophage functions within the lesions. However, this study provided no data supporting a causal role for SOCS in atherogenesis. The first evidence that demonstrated a causal link between SOCS and atherosclerosis is recently provided by Yamamoto and colleagues (62) who indicated that the absence of SOCS-3 in macrophages of $ApoE^{-/-}$ mice decreases atherosclerosis (62). This might be partially related to the inhibitory effect of SOCS3 on IL-6-induced STAT3 activation. Niemand et al., indicated that in the absence of SOCS3, IL-6 has anti-inflammatory effects in macrophages (63).

SOCS1 could have anti-atherogenic effects, especially by inhibiting IFNγ signaling in macrophages and consequently inhibition of the production of the pro-atherogenic molecules CD40 and TNFα, as well as adhesion molecules*. In sum, evidence is emerging from the literature that SOCS-1 and SOCS-3 may be mediators of several disease states, suggesting that molecules that modify or mimic their function may be useful therapeutics (63).*

SOCS and increased CVD in CRF

Inflammation is induced in CRF by overproduction of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6 by chronically active mononuclear cells or by decreased clearance of inflammatory cytokines. These cytokines are potent SOCS inducers in different cell types (64). It is therefore conceivable that in CRF, SOCS is increased in circulating cells and endothelial cells that are in direct contact with ongoing systemic inflammation. In CRF, a few scenarios could be predicted, which might exist simultaneously; 1) increased inflammation causes increased distinct SOCS expression, which could dampen cell response to pro-inflammatory cytokines that signal through Jak/STAT, 2) Increased SOCS could dampen anti-inflammatory cytokines that signal through Jak/STAT. 3) the absence or insufficient SOCS expression, despite increased inflammation, could result in uninhibited cytokine signaling through Jak/STAT with detrimental consequences. 4) increased SOCS might differentially modulate pro and anti-inflammatory signals initiated by a distinct pleiotropic cytokine.

Increased SOCS expression could be involved in pathogenesis of inflammatory diseases. For instance increased SOCS1 and SOCS3 expression could be involved in metabolic syndrome, which is related to diminished STAT3 activation (65). Furthermore, increased SOCS1 expression in cardiac myocyte inhibits enterovirus-induced signaling of Jak/STAT, which is accompanied by increased viral replication, cardiomyopathy, and mortality in this model (66). Interestingly, inhibition of SOCS1 in the same mice model increased myocyte resistance to the acute cardiac injury caused by enteroviral infection (66). These findings indicate that increased SOCS expression within cells could inhibit protective signals through Jak/STAT, resulting in damaging effects.

Another scenario in CRF is the absence or insufficient induction of distinct SOCS expression that could allow harmful signals (that pass through Jak/STAT) to act without being

interrupted. For instance, insufficient SOCS1 expression results in autoimmune diseases, which is related to prolonged activation of STAT molecules (49). Similarly, severity of acute arthritis is much stronger in the absence of SOCS1 (67). SOCS1 is specially important since it can modulate responses to different cytokines including IFNγ, IL12 and IL-4 (49). Furthermore, joint arthritis in SOCS3$^{-/-}$ mice model is particularly severe, which is related to enhanced responsiveness to granulocyte-colony stimulation factor (G-CSF) and IL-6 (68). Therefore, insufficient SOCS expression is also involved in dysregulation of Jak/STAT pathway and consequently in its detrimental effects.

In CRF patients, increased inflammatory cytokines is accompanied by a state of resistance to growth hormone (GH), erythropoietin (Epo), and insulin (69-71). Interestingly, these factors exert their actions via the Jak/STAT pathway, and therefore their signals can be inhibited through induction of SOCS proteins. Possible increased SOCS expression in CRF could, therefore, be one of the mechanisms involved in GH, Epo, or insulin resistance. Indeed, it has been demonstrated that dysregulation of Jak/STAT signaling, by increased levels of SOCS2 and SOCS3, contribute to the GH resistance in rats with experimental CRF (72). Similarly, CIS-1 and SOCS3 might be involved in Epo resistance (73, 74). In particular, CIS-1 is an important inhibitor of Epo signaling. This notion is based on the observation that in CIS-1$^{-/-}$ mice, T-cells and hematopoietic cells are hyper-responsive to Epo (74). Impaired Jak/STAT signaling by increased SOCS expression has also been correlated with insulin-resistance (65).

Summary

In sum, the uremic condition in CRF is accompanied by low-grade inflammation. In this regard, production of inflammatory cytokines by activated mononuclear cells is central. Inflammation in CRF is accompanied by inhibited transmission of beneficial factors such as Epo, insulin and GH. As both EPO and GH also signals through the Jak/STAT pathway, increased SOCS could possibly be involved in inhibition of Epo and GH signaling (72, 73). Simultaneously, increased production of pro-inflammatory cytokines, or insufficient SOCS expression to limit the action of pro-inflammatory cytokines, causes further activation of mononuclear, and endothelial cells (that are exposed to increased inflammation) and consequently increased atherosclerosis in CRF (fig. 2). This suggests that under uremic

conditions there is an imbalance between pro and anti-inflammatory signals in favor of pro-inflammatory pathways. Dysregulation in Jak/STAT & SOCS pathway, which could be involved in increased inflammation and consequently facilitated atherosclerosis in renal patients, has not been studied previously. We suggest that increased SOCS expression could result in resistance to some protective signals through Jak/STAT pathway, while increased SOCS might not be in proportion to the elevated pro-inflammatory cytokines that are consequently insufficiently limited in their actions. Therefore, insufficient SOCS expression in CRF could be the underlying cause of continuous inflammation by prolonged activation of Jak/STAT pathway by pro-inflammatory cytokines. At the same time, increased SOCS might differentially modulate pro and anti-inflammatory signals initiated by the same pleiotropic cytokine (like IL-6), thereby shifting its biological function towards a more pro-inflammatory nature.

Hypothesis

The central hypothesis in this thesis is that uremic conditions in CRF cause increased SOCS expression in MNC (and potentially in EC). Furthermore, increased SOCS expression in monocytes or lymphocytes could reflect whether cells experience inflammation or not, and, in relation to other markers and risk factors of CVD, could represent a new intracellular marker of inflammation-related CVD in CRF. In addition, we suggest that increased SOCS expression could cause an imbalance between components of JAK/STAT, so that harmful signals are left uninhibited and beneficial signals are dampened. This leads to an increased inflammatory state (Fig. 2).

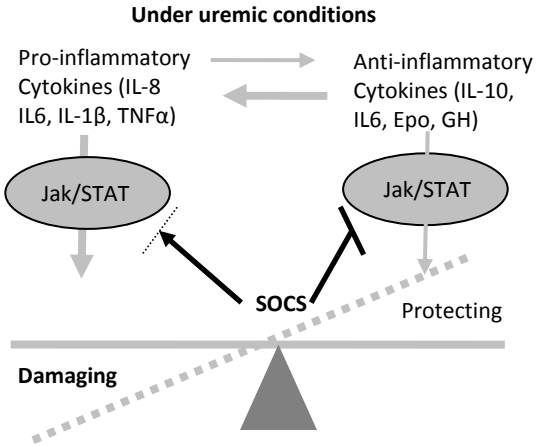


Fig. 2. Differential modulation of Jak/STAT pathway by SOCS. Increased production of pro-inflammatory cytokines in most cases is resolved by production of anti-inflammatory cytokines that antagonize the function of pro-inflammatory cytokines. However, higher level of pro-inflammatory cytokines shifts the balance toward a pro-inflammatory state in CRF. Furthermore, increased SOCS expression could be involved in resistance to anti-inflammatory signals, while the level of SOCS might not be in proportion to increased pro-inflammatory cytokines in CRF.

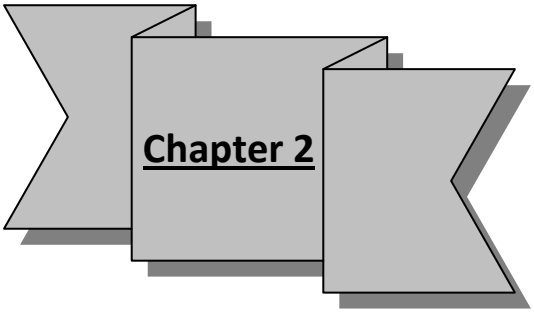
Outline of this thesis

The role of inflammation as a cause of atherosclerotic CVD in renal patients has been extensively studied (1, 17). This is not true for the inducible inhibitors of cytokine signaling; SOCS, which play a crucial role in pathogenesis of other inflammatory diseases (49, 64). This thesis is the first attempt to make a link between SOCS expression, inflammation and CVD in CRF patients. We investigated the increased inflammation in CKD **(chapter 2)** and ESRD **(chapter 3)** patients by characterizing the plasma IL-6, TNFα and CRP. In addition the pattern of SOCS expression in monocytes and lymphocytes and its relation to inflammation and other CVD risk factors will be demonstrated. Furthermore, we will discuss the value of SOCS as a new intracellular marker of inflammation-related CVD in CRF patients.

CRF is accompanied by increased levels of some pro-inflammatory cytokines and inhibition of some protective stimuli such as Epo and GH (69, 72). Furthermore, increased inflammation is accompanied by endothelial dysfunction (that could explain increased atherosclerosis) in CRF. This indicates that damaging signals are present, while protective signals are dampened. This could be (partially) explained by the fact that cytokines can potentiate or inhibit signaling by other cytokines. In this regard SOCS might play a crucial role. In **Chapter 4**, the cross-talk between two important atherogenic cytokines IFNγ and IL-6 (that increased in CRF) has been studied in endothelial cells. Similar to some other inflammatory cytokines (63), IFNγ inhibited IL-6-induced STAT3 activation, which is the main transcription factor in IL-6 signaling.

IL-6 has both pro and anti-inflammatory effects. Therefore, it is important to understand whether the inhibitory effect of IFNγ on IL-6, similarly or differentially, affects the pro and anti-inflammatory effects of IL-6. In **chapter 5**, using gene expression profiling, we studied how cross-talk between IFNγ and IL-6 affected downstream target genes involved in pro- or anti-inflammatory pathways.

Similar to other studies (75), our data indicated that, in addition to STAT3, other transcription factors are involved in IL-6 signaling. As IL-6 is able to signal through STAT1 in $STAT3^{-/-}$ cells, we suggested that STAT1 could be a potential transcription factor under IL-6 signaling cascade. In **chapter 6**, we presented preliminary data obtained from wild type and $STAT1^{-/-}$ mice, concerning the role of STAT1 in IL-6 signaling.



Increased expression of SOCS3 in monocytes and SOCS1 in lymphocytes correlates with progressive loss of renal function and cardiovascular risk factors in chronic kidney disease

M. Mehdi Rastmanesh[1], Hans A.R. Bluyssen[2], Jaap A. Joles[1], Peter Boer[1], Nel Willekes[1], Branko Braam[1,3,4]

[1] Department of Nephrology and Hypertension, University Medical Center Utrecht, the Netherlands. [2] Institute of Molecular Biology and Biotechnology, Adam Mickiewicz University, Poznan, Poland. [3] Division of Nephrology and Immunology, Dept. Medicine, Univ. of Alberta, Edmonton, Canada and [4] Dept. Physiology, Univ. of Alberta, Edmonton, Canada.

Eur. J. Pharmacol. 2008 Sep ; 593(1-3):99-104

Abstract

Inflammation, an independent cardiovascular disease risk factor is common in patients with chronic kidney disease. Suppressors of Cytokine Signaling (SOCS) are induced by cytokines in a variety of cells and modulate inflammatory responses. We hypothesized that in chronic kidney disease (CKD), SOCS expression in peripheral blood mononuclear cells (PBMC) is increased, and related to inflammation and renal function. We also tested correlations between SOCS expression and biomarkers and risk factors of cardiovascular disease. Whether monocytes and lymphocytes differentially respond to interleukin-6 (IL-6) was also tested *ex-vivo*.

Monocytes and lymphocytes were isolated from peripheral blood of chronic kidney disease patients (n=9) and controls (n=11). In three other healthy subjects, whole blood was incubated with IL-6 before cell isolation. SOCS expression was assessed by real-time quantitative PCR. Plasma cytokines were measured as well as pulse wave velocity.

SOCS3 expression was increased in monocytes and SOCS1 in lymphocytes along with increased plasma levels of IL-6 and tumor necrosis factor-α (TNFα) in chronic kidney disease patients. While monocyte SOCS3 correlated with glomerular filtration rate, urea and diastolic blood pressure, lymphocyte SOCS1 correlated with TNFα, pulse wave velocity and systolic blood pressure. IL-6 stimulation of whole blood caused expression of different SOCS genes in monocytes and lymphocytes.

Increased expression of SOCS3 in monocytes versus SOCS1 in lymphocytes coincided with elevated plasma levels of IL-6 and TNFα, suggesting that these cell types process the uremic milieu differently. This could reflect cell-specific responses to inflammation, as supported by our *ex vivo* study. Moreover, increased SOCS expression in peripheral blood mononuclear cells correlated with decreased renal function. Since chronic kidney disease predisposes to cardiovascular disease, we speculate that increased SOCS expression in peripheral blood mononuclear cells could be a new marker of cardiovascular disease in chronic kidney disease patients.

Introduction

Cardiovascular disease is the major cause of morbidity and mortality in patients with chronic kidney disease (76, 77). In addition to traditional risk factors (e.g. hypertension and dyslipidemia) (76, 78), chronic kidney disease forms an independent risk factor for cardiovascular disease. Inflammation and oxidative stress are considered central in the pathogenesis.

Many inflammatory cytokines signal through the Janus Kinase / Signal Transducer and Activator of Transcription (Jak/STAT) pathway (79, 80). Suppressors of cytokine signaling (SOCS) proteins (Cytokine-inducible SH2-containing protein-1, CIS-1, and SOCS-1 to -7) regulate strength and duration of the inflammatory signaling cascade by inhibiting the Jak/STAT signaling (48, 81). Each cytokine specifically induces one or more SOCS proteins, which can inhibit the signal initiated by the cytokine itself and potentially by other cytokines (82). Thus, SOCS proteins are not only modulators of cytokine signaling, but also they reflect the activation of intracellular cytokine pathways. The physiological importance of the SOCS proteins is illustrated by the strong macrophage infiltration in $SOCS1^{-/-}$ mice (83) and the erythrocytosis in $SOCS3^{-/-}$ mice (84). Moreover, SOCS1 and SOCS3 are frequently increased in different inflammatory diseases (68).

Chronic kidney disease is associated with increased production of pro-inflammatory cytokines interleukin 6 (IL-6) and tumor necrosis factor α (TNFα) (19, 85) and with activation of peripheral blood mononuclear cells (23, 32, 85-87). Inflammatory cytokines in turn regulate the adhesion of monocyte to endothelium (88) and skew monocyte differentiation to macrophages (89). SOCS proteins tightly regulate the action of those cytokines. Furthermore, SOCS are involved in differentiation, cytokine production and survival of T-cells (90, 91). Whether chronic kidney disease affects monocyte and lymphocyte SOCS expression and how SOCS relate to residual renal function, inflammatory cytokines, and risk factors of cardiovascular disease in chronic kidney disease is unknown.

We therefore hypothesized that in chronic kidney disease SOCS expression in peripheral blood mononuclear cells is increased, and related to inflammation and renal function. We also tested correlations between SOCS expression and biomarkers and risk

factors of cardiovascular disease. Finally, we tested whether monocytes and lymphocyte differentially respond to IL-6 ex-vivo.

Material and method

Subjects, blood samples and pulse wave velocity

Nine chronic kidney disease patients in Stage 3-4 of renal failure (according to the classification of Kidney Disease Outcomes Quality Initiative (K/DOQI)) and 11 healthy controls were included. None of the chronic kidney disease patients was dialyzed. Subjects with diabetes, hepatitis, infection, cancer, recent surgery or on immunosuppressive medication were excluded. Blood pressure and body mass index were assessed. Pulse wave velocity was measured as described previously (92). Fasting blood (50 ml) was sampled in EDTA-anticoagulated tubes. Hemoglobin was measured with a Multiparameter Haematology Analyzer, Cell Dyn 1700 (Abbott, Hoofddorp, the Netherlands). Serum samples were analyzed for albumin, blood lipids, C-reactive protein (CRP; CRP values of <5 considered 4.99 and of <7 considered 6.99), glucose, creatinine, and urea using routine laboratory methods. Three healthy individuals donated blood for ex-vivo stimulation with IL-6. The Medical Ethics Committee of the University Medical Center Utrecht approved the study. Informed consent was obtained from all participants.

Plasma cytokines

Plasma levels of IL-6, IL-10, and TNFα were measured using highly sensitive colorimetric sandwich ELISA kit (R&D Systems) according to the manufacturer's instructions. The lower detection limits were 0.16-5 pg/ml for IL-6, 0.8-25 pg/ml for IL-10, and 0.5-16 pg/ml for TNFα. All samples were measured in duplicate.

Isolation of peripheral blood mononuclear cells, monocytes and lymphocytes

Peripheral blood mononuclear cells were isolated using Ficoll® (Amerhsam) according to the manufacturer's instructions. Monocytes were isolated using indirect magnetic labeling system (MACS®, Miltenyi Biotec) for the isolation of untouched monocytes from human peripheral blood mononuclear cells. Non-monocytes i.e. T cells, natural killer

cells (NK), B cells, dendritic cells and basophils were indirectly magnetically labeled using a cocktail of biotin-conjugated antibodies against CD3, CD7, CD16, CD19, CD56, CD123, glycophorin A, and Anti-Biotin microbeads (93). Since lymphocytes form the majority of non-monocytic mononuclear cells, we refer to this fraction as lymphocytes (94). Cell separation procedure was performed on ice. For ex-vivo experiments, whole blood was incubated with 20 U/ml IL-6 (Sigma) for 60 min at room temperature before cell isolation was performed.

Quantitative PCR

Total RNA was isolated using TRIzol® (Gibco). The quality of isolated RNA was tested using a Bio-Analyzer™ system (Agilent). RNA from lymphocytes of one chronic kidney disease subject was excluded because of RNA degradation. One µg RNA was reverse transcribed (iScript® cDNA synthesis Kit, Bio-Rad). Gene expression was assessed by real-time PCR using pre-designed SOCS1, SOCS2, SOCS3, CIS1, GAPDH and 18S primers (Applied Biosystems TaqMan® Assays-on-Demand™) on an Applied Biosystems 7900 HT Fast Real-Time PCR System. Expression of each target gene in each subject was normalized to endogenous controls GAPDH+18S (ΔCT) and related to a reference (ΔΔCT), being RNA from untreated peripheral blood mononuclear cells of a healthy volunteer. Gene expression is expressed as $(2^{-\Delta\Delta CT} * 100) \pm$ S.D. All samples were tested in duplicate.

Statistics

Results are expressed as mean ± S.D. Data were compared using Student's t-test, or, if data were not distributed normally, the Mann-Whitney rank sum test. Correlation coefficients were calculated by Pearson's product moment correlation test, or, if the data were not distributed normally, the Spearman's rank order correlation test. All statistical tests were performed with SigmaStat (version 3.5, Systat Software Inc, San Jose, Ca). $P < 0.05$ was considered to be significant.

Results

Clinical characteristics

In chronic kidney disease patients, diastolic blood pressure was increased ($P < 0.05$), whereas systolic blood pressure was not (table 1). Furthermore, hemoglobin and albumin were significantly lower in patients compared to controls ($P < 0.05$). Etiologies of chronic kidney disease were: unknown (n=3), hypertension (n=3), glomerulosclerosis (n=2), autosomal dominant polycystic kidney disease (n=1). No patient received erythropoietin. Medication is summarized in table 2.

Table 1. General and biochemical variables of chronic kidney disease and control subjects

Variables	Controls	Chronic kidney disease patients
N	11	9
Age (year)	56.4 ± 12.2	64.1 ± 9.6
F : M	6 : 5	6 : 3
Body mass index (kg/m^2)	25.3 ± 3.4	25.1 ± 3.9
Urea (mmol/L)	5.6 ± 1.2	14.7 ± 6.2[a]
Creatinine (µmol/L)	81 ± 8.7	191 ± 62[a]
Estimated GFR (ml/min/1.73 m^2)	78 ± 9.0	30 ± 8[a]
Systolic blood pressure mmHg	121 ± 12.7	138 ± 31
Diastolic blood pressure mmHg	73 ± 5.7	81 ± 8[b]
Pulse wave velocity (m/s)	6.6 ± 1.8	9.9 ± 2.6[b]
Cholesterol (mmol/L)	5.6 ± 0.9	5.2 ± 0.8
HDL (mmol/L)	1.4 ± 0.5	1.4 ± 0.4
LDL (mmol/L)	3.5 ± 0.8	3.1 ± 0.4
Albumin (g/L)	45.7 ± 2.2	42.9 ± 3.3[b]
Glucose (mmol/L)	5.3 ± 0.5	5.4 ± 0.5
Triglycerides (mmol/L)	1.3 ± 0.5	1.5 ± 0.5
Hemoglobin (mmol/L)	9.5 ± 0.9	8.0 ± 1.2[b]

[a] $P < 0.001$, [b] $P < 0.05$

Table 2. Summary of medications of chronic kidney disease and control subjects

Medications	Controls	Chronic kidney disease patients
Antihypertensives	None	7 of 9
Diuretics	None	7 of 9
Statins	None	6 of 9
Folic acid	None	4 of 9
Anticoagulants	None	2 of 9

Plasma IL6, TNFα, IL-10 and CRP

Confirming previous studies (95) we found increased plasma levels of both IL-6 and TNFα in chronic kidney disease subjects versus controls ($P < 0.01$; fig. 1A). IL-10 (data not shown) and CRP (fig. 1A) levels were unchanged in chronic kidney disease patients. A significant negative correlation between IL-6 and glomerular filtration rate could be demonstrated ($r = -0.69$, $P < 0.05$; Fig. 1B), this was not the case for TNFα.

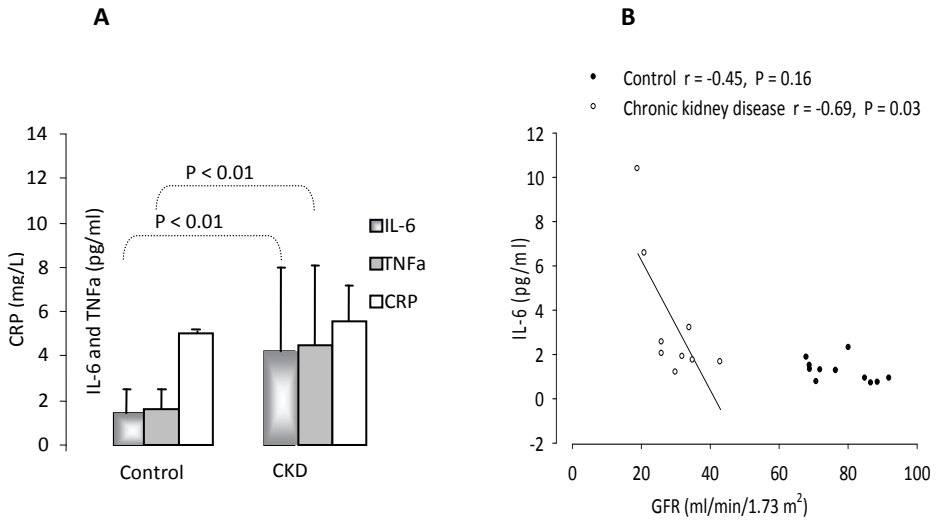


Fig. 1. Increased plasma level of inflammatory cytokines in chronic kidney disease patients. Plasma levels of IL-6, TNFα and CRP in healthy controls and chronic kidney disease subjects were measured as described at material and methods (A). Correlation between IL-6 and glomerular filtration rate (GFR) were calculated by spearman rank order correlation test (B). The values are the mean ± S.D. of 11 controls and 9 chronic kidney disease patients. $P < 0.05$ was considered significant.

Differential SOCS gene expression in lymphocytes and monocytes

SOCS3, but not CIS1 and SOCS1 expression was significantly increased in monocytes of chronic kidney disease patients versus controls ($P < 0.01$; fig. 2A). In contrast, SOCS1, but not CIS1 and SOCS3 expression was increased in lymphocytes of patients ($P < 0.05$; fig. 2B). The highly variable expression of SOCS2 in both cell types prevented further analyses. A significant relationship between monocyte SOCS3 and estimated glomerular filtration rate ($r = -0.70$, $P < 0.05$; fig. 3A) as well as urea ($r = 0.80$, $P < 0.01$; fig. 3B) could be demonstrated. Furthermore, a significant negative correlation was found between diastolic blood pressure and monocyte SOCS3 ($r = -0.69$, $P < 0.05$; fig. 3C). This was not the case for lymphocyte

SOCS1. In contrast lymphocyte SOCS1 was significantly related to plasma TNFα, pulse wave velocity (r= 0.682, P < 0.05 and r = 0.857, P < 0.05, respectively; data not shown) and systolic blood pressure (r = 0.78, P < 0.05; fig. 4). Together these data link peripheral blood mononuclear cells SOCS expression in chronic kidney disease to the known risk factors and markers of cardiovascular disease and suggest the potential of SOCS as a new intracellular marker of cardiovascular disease in chronic kidney disease patients.

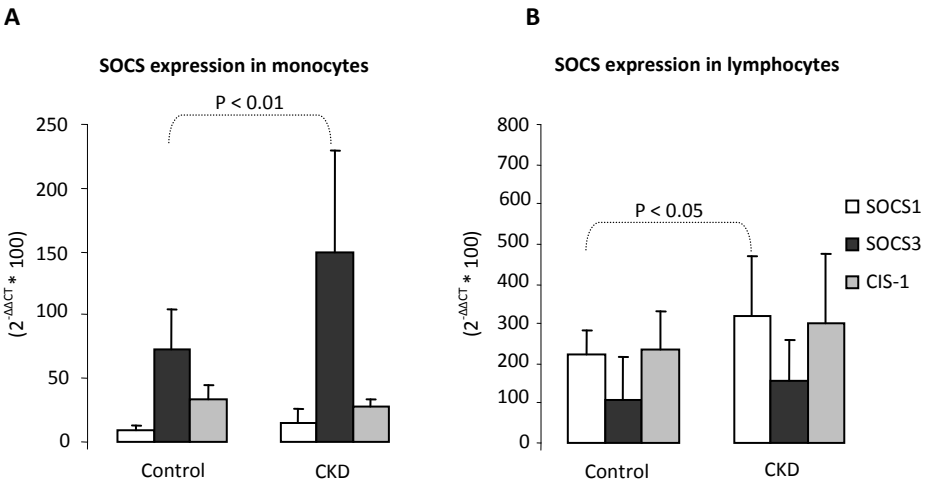


Fig. 2. Increased SOCS expression in monocytes and lymphocytes of chronic kidney disease patients. SOCS1, SOC3 and CIS-1 gene expression in monocytes (A) and in lymphocytes (B) of healthy controls and chronic kidney disease patients were measured using quantitative real time PCR. The values are mean ($2^{-\Delta\Delta CT}$ * 100) ± S.D. of gene expression in samples from 11 controls and 9 chronic kidney disease subjects. RNA from lymphocytes of one chronic kidney disease subject was excluded because of RNA degradation. P < 0.05 was considered significant.

Monocytes and lymphocytes differentially express SOCS genes in response to IL-6.

As underscored by SOCS1/SOCS3 and CIS1/SOCS3 ratios (fig. 4A), SOCS1 and CIS1 expression was significantly higher in lymphocytes as compared to monocytes of both chronic kidney disease and control groups (P < 0.001), while SOCS3 expression was of comparable level. Our data further suggest that lymphocytes and monocytes differentially respond to uremia-related inflammation, as indicated by differential SOCS expression (fig. 2A and 2B). To obtain further evidence for this, we incubated whole blood from three healthy subjects with IL-6 for 1 hour and measured SOCS1, SOCS3 and CIS-1 expression in both monocytes and

lymphocytes. IL-6 only caused increased expression of SOCS3 in monocytes ($P < 0.001$), however, increased expression of both SOCS1 and SOCS3 in lymphocytes ($P < 0.05$, and $P < 0.01$ respectively; fig. 4C). Furthermore, SOCS3 induction in monocytes was much stronger than in lymphocytes ($P < 0.01$) (fig. 4C). Together, these ex-vivo experiments indicate that IL-6 can elicit differential responses in peripheral blood monocytes and lymphocytes, in line with our in-vivo data.

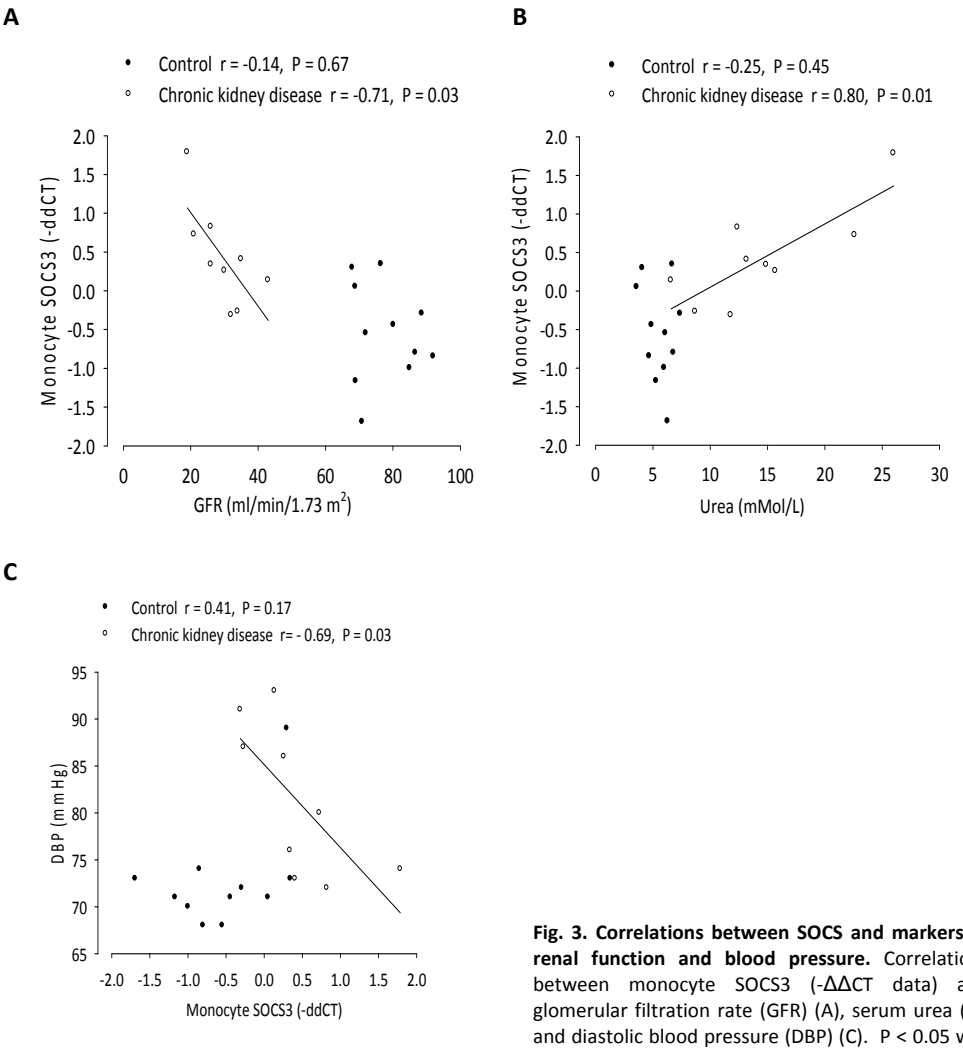


Fig. 3. Correlations between SOCS and markers of renal function and blood pressure. Correlations between monocyte SOCS3 (-ΔΔCT data) and glomerular filtration rate (GFR) (A), serum urea (B), and diastolic blood pressure (DBP) (C). $P < 0.05$ was considered significant.

Discussion

Our study revealed increased expression of SOCS3 in monocytes and of SOCS1 in lymphocytes of chronic kidney disease patients accompanied by increased plasma levels of the inflammatory cytokines IL-6 and TNFα. Interestingly, increased monocyte SOCS3 significantly correlated with progressive loss of renal function, measured by estimated glomerular filtration rate and urea. Moreover, lymphocyte SOCS1 was correlated with other known markers and risk factors of cardiovascular disease, TNFα, systolic blood pressure and pulse wave velocity. In particular, systolic blood pressure and pulse wave velocity have been related to arterial stiffness (96), and the latter to the outcome of end-stage renal disease (97). Therefore, these results link peripheral blood mononuclear cells SOCS expression in patients with chronic kidney disease to known markers and risk factors of cardiovascular disease and indicate SOCS expression as potential new intracellular marker of cardiovascular disease in chronic kidney disease patients. In analogy, loss of SOCS2 expression has recently been associated with cell proliferation and tumor growth in breast carcinoma (58).

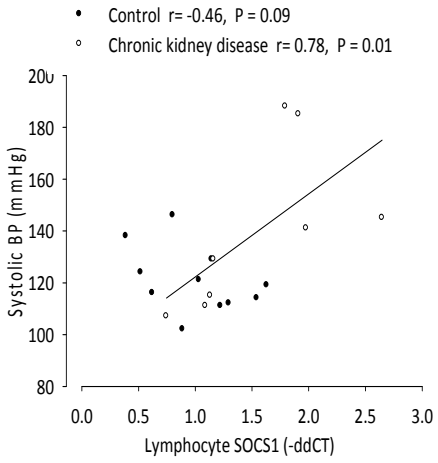


Fig. 4. Correlation between SOCS and inflammation and cardiovascular disease risk factors. Correlation between lymphocyte SOCS1 (-ΔΔCT data) and systolic blood pressure (SBP). $P < 0.05$ was considered significant.

Monocytes are involved in initiation (98) and progression of atherosclerosis. Increased monocyte SOCS3 could indicate enhanced activity of inflammatory cytokines such as IL1-β, TNFα, IL-6, IFNγ and IL-10 (82), but also of Angiotensin II (Ang II) (99) and LPS (100). Medication such as statins and Ang II inhibitors could also modulate SOCS3 expression in monocytes (53). In this study, chronic kidney disease patients exhibited increased levels of IL-

6 and TNFα. However, even though our ex-vivo data clearly demonstrated a potential role of IL-6 in SOCS3 induction in monocytes, no significant correlation could be detected between monocyte SOCS3 with plasma IL-6 or with TNFα. As such, SOCS3 gene expression may reflect how the overall inflammatory factors and disturbances in the internal environment in chronic kidney disease affect the monocytes. In accordance is the correlation between monocyte SOCS3 and estimated glomerular filtration rate and serum urea.

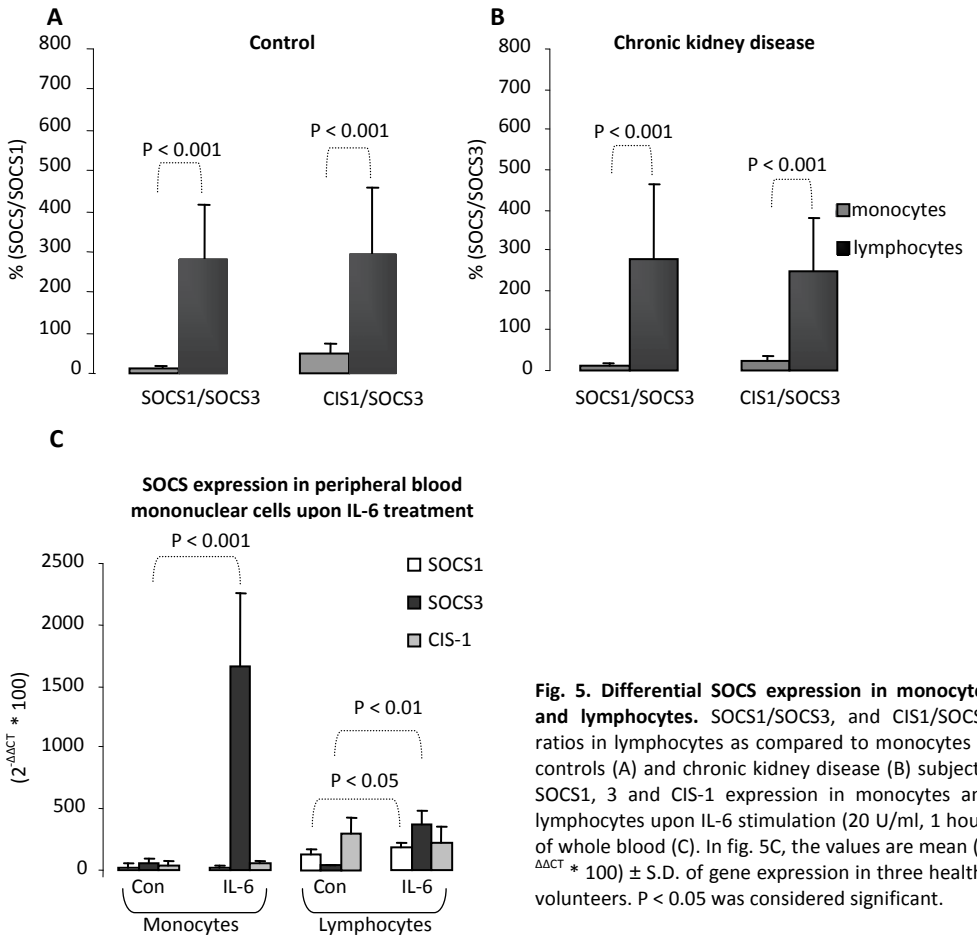


Fig. 5. Differential SOCS expression in monocytes and lymphocytes. SOCS1/SOCS3, and CIS1/SOCS3 ratios in lymphocytes as compared to monocytes in controls (A) and chronic kidney disease (B) subjects. SOCS1, 3 and CIS-1 expression in monocytes and lymphocytes upon IL-6 stimulation (20 U/ml, 1 hour) of whole blood (C). In fig. 5C, the values are mean ($2^{-\Delta\Delta CT}$ * 100) ± S.D. of gene expression in three healthy volunteers. P < 0.05 was considered significant.

Beyond simply being a new intracellular biomarker of cardiovascular disease, increased SOCS3 may inhibit or modulate cytokine signaling. Recently, it was demonstrated that SOCS3 predominantly plays a negative regulatory role in STAT3 activation and in biologic

responses to IL-6 (101). Interestingly, in mice lacking SOCS-3 in macrophages, IL-6 has anti-inflammatory effects and inhibits production of TNF-α and IL-12 from macrophages (101). Moreover, a recent study by Yamamoto T. and colleagues indicates that the absence of SOCS-3 in macrophages of apolipoprotein-E knock out ($ApoE^{-/-}$) mice decreases atherosclerosis (62). This studies strongly point to the pro-inflammatory and pro-atherogenic effects of SOCS3. Other studies suggest a role for SOCS3 in inhibition of insulin (102), growth hormone (103) and Erythropoietin (73) signaling.

However, SOCS3 could have also anti-inflammatory effects. For instance, anti-inflammatory effects of IL-10 and statins might be related to the induction of SOCS3 (104, 105). Therefore, this remains an exciting research area for follow up studies.

Lymphocytes have been implicated in the atherosclerotic process in a variety of ways. Subsets of T helper (Th) cells are important producers of pro-inflammatory cytokines; Th1 cells produce pro-inflammatory cytokines like IFNγ, TNFα and IL-12 and -18, all of which have been shown to promote atherogenesis (106). Th2 cells produce anti-inflammatory cytokines like IL-10 and antagonize Th1 (107). SOCS1 is specifically expressed in Th1 cells. Since SOCS1 expression in Th1 cells specifically inhibits the growth inhibitory effects of IFNγ on these cells, but not on Th2 cells (108), it is possible that increased lymphocyte SOCS1 in chronic kidney disease skews the immune response to the Th1 type in these patients (109), in favor of inflammation and atherosclerosis development.

In contrast to monocyte SOCS3, increased lymphocyte SOCS1 was not correlated with loss of renal function or diastolic blood pressure, but it was positively correlated with plasma TNFα, systolic blood pressure and pulse wave velocity. Together, these observations provide evidence to suggest that these two mononuclear cell types differentially process uremia-related inflammation. In line with these considerations are our ex-vivo experiments. Using whole blood, IL-6 increased SOCS3 expression in monocytes as well as lymphocytes but only increased SOCS1 expression in lymphocytes. Although SOCS3 was induced in both cell types, IL-6-induced SOCS3 expression in monocytes was substantially higher than in lymphocytes.

Unfortunately, we were not able to recruit patients with cardiovascular disease without renal failure. Availability of such data would allow us to dissect whether changes in SOCS

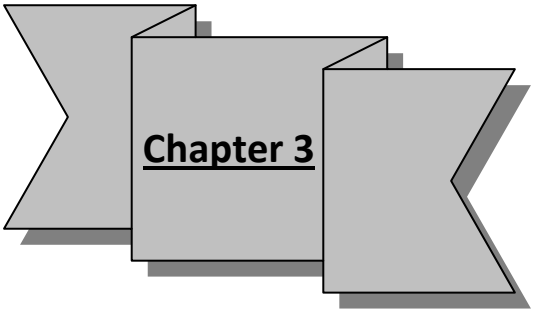
expression in peripheral blood mononuclear cells is specific for chronic kidney disease patients or can also be seen in cardiovascular disease patients with normal kidney function. However, we believe that increased systemic inflammation, an additional cardiovascular disease risk factor in chronic kidney disease patients, is the key factor in affecting SOCS expression in peripheral blood mononuclear cells. This idea is supported by a previous report, which indicated that inflammatory cytokines are increased in renal patients in comparison to cardiovascular disease patients with normal renal function (7). Theoretically medication could affect SOCS expression in peripheral blood mononuclear cells. Although this should be addressed in an adequate number of patients, preliminary data in 3 chronic kidney disease patients indicated that medical intervention for four weeks did not affect SOCS expression in peripheral blood mononuclear cells significantly (data not shown).

In sum our data show increased monocyte SOCS3 and lymphocyte SOCS1 in chronic kidney disease patients in relation to cardiovascular disease risk factors including inflammation. This is the first report to introduce SOCS expression in peripheral blood mononuclear cells as a new marker for cardiovascular disease in chronic kidney disease patients. However, this study needs to be confirmed in a larger chronic kidney disease cohort. Understanding whether changes in SOCS expression in peripheral blood mononuclear cells of chronic kidney disease patients is accompanied by endothelial cell dysfunction and atherosclerotic plaque formation is a necessary knowledge to confirm the value of SOCS expression in peripheral blood mononuclear cells of chronic kidney disease patients as a new marker of cardiovascular disease. In this context, increased SOCS expression in peripheral blood mononuclear cells might also be an indicator for increased SOCS expression in the vascular wall. In fact the presence of SOCS1 and SOCS3 in atherosclerotic plaques has recently been demonstrated (61). Furthermore, characterizing the role of SOCS1 and SOCS3 in more detail in the development of cardiovascular disease in chronic kidney disease patients may well provide new therapeutic targets for cardiovascular disease. We were unable to measure SOCS proteins in peripheral blood mononuclear cells because of technical difficulties. Hence, this should be addressed in follow up studies to confirm our data at the protein level. Once confirmed, SOCS could become a marker of occult cardiovascular disease in chronic kidney disease patients without symptoms of cardiovascular disease. It is also noteworthy, that our

data indicated that plasma cytokines and SOCS expression were predominantly increased in patients in stage four (15 < GFR < 30 ml/min/1.73 m^2) of renal disease (data not shown), pointing to the importance of therapeutic intervention in early stages of renal failure to delay increased inflammation, which might delay cardiovascular disease progress.

Acknowledgements

We gratefully thank Dr. P.J. Blankestijn, Dr. W.H. Boer, Dr. A.V. van Zuilen and Dr. P.F. Vos for enrolling chronic kidney disease patients and C. Slee-Veenma for blood sampling. This work was supported by grants from Dutch kidney foundation (NSN C03-2062) and a grant by the Genzyme Renal Innovations Program (GRIP).



Increased SOCS expression in peripheral blood mononuclear cells of end stage renal disease patients is related to inflammation and dialysis modality

M. Mehdi Rastmanesh[1], Branko Braam[1,2,3], Jaap A. Joles[1], Peter Boer[1], Hans A.R. Bluyssen[4].

[1] Department of Nephrology and Hypertension, University Medical Center Utrecht, the Netherlands. [2] Division of Nephrology and Immunology, Dept. Medicine, Univ. of Alberta, Edmonton, Canada and [3] Dept. Physiology, Univ. of Alberta, Edmonton, Canada. [4] Institute of Molecular Biology and Biotechnology, Adam Mickiewicz University, Poznan, Poland,

In press

Abstract

Inflammation is a characteristic of cardiovascular disease and is increased in end-stage renal disease. Suppressors of cytokine signaling (SOCS) inhibit and reflect activation of intracellular inflammatory pathways. We hypothesized that SOCS expression in peripheral blood mononuclear cells of end stage renal disease patients is increased. Whether SOCS expression in peripheral blood mononuclear cells is related to inflammation, dialysis, and dialysis modality was investigated.

Monocytes and lymphocytes were isolated from peripheral blood mononuclear cells of patients not on dialysis (n=8), on peritoneal dialysis (n=8), on hemodialysis (n=14) and of healthy control (n=15) subjects. SOCS expression was assessed by real-time quantitative PCR and plasma cytokines by ELISA.

In end stage renal disease patients monocyte SOCS1, and lymphocytes SOCS1 and cytokine-inducible SH2 containing protein-1 (CIS-1) gene expression were increased along with increased plasma levels of interleukin (IL)-6, tumor necrosis factor (TNF)α ,and C-reactive protein (CRP). Monocyte SOCS1 correlated with IL-6. Lymphocyte CIS-1 was increased in non-dialysis and peritoneal dialysis but not in hemodialysis patients. Lymphocyte CIS-1 in peritoneal dialysis patients correlated with plasma TNFα.

Despite the relatively low number of patients studied we observed increased expression of SOCS1 in both monocytes and lymphocytes, and of CIS-1 solely in lymphocytes of the patients. SOCS expression was related to increased systemic inflammation, illustrated by a significant correlation between monocyte SOCS1 and plasma IL-6. SOCS expression in peripheral blood mononuclear cells was also affected by hemodialysis, indicated by increased lymphocyte CIS-1 in non-dialysis and peritoneal dialysis but not in hemodialysis patients. We suggest that increased SOCS expression in peripheral blood mononuclear cells of end stage renal disease patients reflects whether and to which extent systemic inflammation activate the intracellular inflammatory pathways.

Introduction

Inflammation is a common feature in end stage renal disease. In fact, C-reactive protein (CRP) and the pro-inflammatory cytokines IL-6, and TNFα are increased at all stages of renal failure (110, 111). Inflammation is known to activate endothelial cells, lymphocytes and monocytes and forms an independent risk factor for cardiovascular disease and mortality in renal patients at all stages of renal failure (88, 110). Interestingly, drugs that diminish inflammation, can reduce atherosclerosis and cardiovascular events (112), pointing to the importance of molecules that inhibit inflammatory signals.

SOCS proteins (SOCS1-7 and CIS-1) are inducible inhibitors of cytokine signaling. Cytokines specifically induce one or more SOCS proteins, which can inhibit the signal initiated by the cytokine itself and potentially by other cytokines (81). Thus, SOCS proteins are not only modulators of cytokine signaling, but also they reflect the activation of intracellular cytokine pathways. Moreover, SOCS have been identified as important regulators of the immune and inflammatory responses, evidenced by the fact that SOCS1 and SOCS3 are frequently increased in inflammatory diseases (61, 68). As such, SOCS expression reflects the initiation of negative feedback responses to an increased inflammatory pressure on cells, resulting in protecting or damaging effects through inhibition of pro- and anti-inflammatory signals, respectively. Differential SOCS expression could therefore be used at the cellular level to test whether patients with end stage renal disease are experiencing increased inflammation.

Renal failure is associated with activation of peripheral blood mononuclear cells. SOCS are involved in differentiation, cytokine production and survival of monocytes and lymphocytes. We recently observed increased SOCS in monocytes and lymphocytes of chronic kidney disease patients that correlated with plasma inflammatory cytokines, renal function and cardiovascular disease markers (113). However, no information is available regarding SOCS expression in monocytes and lymphocytes of end stage renal disease patients and its relation to inflammation, dialysis modality, and markers of cardiovascular disease. We hypothesized that SOCS expression in monocytes and lymphocytes of end stage renal disease patients is increased, related to inflammation, dependent on whether dialysis is initiated, and on which dialysis modality is applied. We also tested the correlation between SOCS expression and cardiovascular disease markers or risk factors.

Materials and methods

Subjects and blood samples

Thirty end stage renal disease patients (non-dialysis, n=8; hemodialysis, n=14; and peritoneal dialysis, n=8) and 15 controls were included. Hemodialysis treatment comprised three 4-hours sessions weekly using synthetic polysulfone membrane and bicarbonate dialysate. Peritoneal dialysis patients were treated with regular continuous ambulatory peritoneal dialysis (n=3) or continuous cycler peritoneal dialysis (n=5). None of the patients had signs of ultrafiltration failure. Exclusion criteria were infection, diabetes mellitus, hepatitis, cancer, recent operation and use of immunosuppressive medication. Blood was sampled (in hemodialysis prior to dialysis and prior to erythropoietin administration) in EDTA-anticoagulated tubes. Plasma was directly isolated and stored at -80ºC for analysis of albumin, lipids, CRP (CRP values of <5 considered 4.99 and of <7 considered 6.99), glucose, creatinine, and urea using routine laboratory methods. Thiobarbituric acid-reactive substances (TBARS) were measured as described previously (114). Hemoglobin was measured with a Multiparameter Haematology Analyzer, Cell Dyn 1700. Glomerular filtration rate (in non-dialysis patients) was calculated using MDRD formula. The study was approved by the Medical Ethics Committee of the Utrecht Medical Center. Informed consent was obtained from all participants.

Plasma cytokines

Plasma IL-6, IL-10, IFNγ, and TNFα levels were measured using colorimetric ELISA kits and IL-2 levels using a chemiluminescent ELISA (R&D Systems). Detection limits were 0.16-5 pg/ml for IL-6, 0.8-25 pg/ml for IL-10, 15.6-500 pg/ml for IFNγ, 0.5-16 pg/ml for TNFα, and 1.7-1250 pg/ml for IL-2.

Isolation of peripheral blood mononuclear cells, monocytes and lymphocytes

Peripheral blood mononuclear cells were isolated using Ficoll® (Amerhsam) according to the manufacturer's instructions. Monocytes were isolated using indirect magnetic labeling system (MACS®, Miltenyi Biotec) for the isolation of untouched monocytes from human peripheral blood mononuclear cells. Non-monocytes i.e. T cells, natural killer

cells (NK), B cells, dendritic cells and basophils were indirectly magnetically labeled using a cocktail of biotin-conjugated antibodies against CD3, CD7, CD16, CD19, CD56, CD123, glycophorin A, and Anti-Biotin microbeads (93). Since lymphocytes form the majority of non-monocytic mononuclear cells, we refer to this fraction as lymphocytes (94). Cell separation procedure was performed on ice.

Quantitative PCR

RNA was isolated using TRIzol® (Gibco). Quality of isolated total RNA was tested using a Bio-Analyzer™ system (Agilent). RNA from two monocyte and two lymphocyte samples of controls and one lymphocyte sample from the non-dialysis group were excluded because of RNA degradation. One µg RNA was reverse transcribed (iScript® cDNA synthesis Kit, Bio-Rad). Gene expression was assessed by real-time PCR using pre-designed SOCS1-3, CIS1, GAPDH and 18S primers (Applied Biosystems TaqMan® Assays-on-Demand™). Expression of each target gene in each subject was normalized to endogenous controls GAPDH+18S (ΔCT) and related to a reference (ΔΔCT), being RNA from untreated peripheral blood mononuclear cells of a healthy volunteer. Gene expression is expressed as ($2^{-\Delta\Delta CT}$ * 100) ± SD.

Statistics

Results are expressed as mean ± SD. Data were compared using Student's t-test, or a one Way ANOVA and the Studentized Newman Keuls post-hoc test, where appropriate. If data were not distributed normally we used the Mann-Whitney rank sum test or Kruskal-Wallis ANOVA on ranks and Dunn's post-hoc test. Correlation coefficients were calculated by Pearson's product moment correlation test (a linear regression test). $P < 0.05$ was considered significant.

Results

General and biochemical data

Systolic (SBP) and diastolic (DBP) blood pressure were increased in end stage renal disease patients (table 1) as compared to healthy controls ($P < 0.001$ and $P < 0.01$ respectively). Total and LDL cholesterol, albumin and hemoglobin (Hb) were decreased ($P < 0.001$). CRP, used as marker of inflammation, was significantly increased in end stage renal disease ($P < 0.01$). This was not the case for TBARS, a marker of oxidative stress ($P = 0.35$). There were no significant differences among the end stage renal disease subgroups except for the SBP, which was significantly higher in non-dialysis (148±28 mmHg) and hemodialysis (147±20 mmHg) groups versus both peritoneal dialysis (113±14 mmHg) and controls (121±12 mmHg) ($P < 0.01$). Etiologies of renal failure were: unknown (n= 9), hypertension (n=5), Lupus erythematosis (n=3), hemolytic uremic syndrome (n=1), nephritic syndrome (n=1), glomerulonephritis (n= 2), glomerulosclerosis (n=2), Alport's syndrome (n=1), Goodpasture's syndrome (n=1), autosomal dominant polycystic kidney disease (n=4), and microscopic polyangitis (n=1). All hemodialysis patients received recombinant EPO (once a week, during hemodialysis), 6 of the 8 peritoneal dialysis patients (once a week), and 3 of the 8 non-dialysis patients (once a month) received recombinant EPO subcutaneously. Medication is summarized in table 2. n represents the number of patients.

Increased monocyte SOCS1 and lymphocyte SOCS1 and CIS-1 in end stage renal disease patients

Expression of SOCS1, but not SOCS3 and CIS-1, was increased in monocytes of end stage renal disease patients versus controls ($P < 0.05$; fig. 1A). In contrast, expression of SOCS1 and CIS-1, but not SOCS3, was increased in lymphocytes of end stage renal disease patients ($P < 0.05$, and $P < 0.01$ respectively; fig. 1B). SOCS2 expression was highly variable in both cell types (not shown). SOCS expression was also analyzed separately according to dialysis modality and to whether dialysis was initiated or not. We found that monocyte SOCS1 was predominantly increased in hemodialysis patients ($P = 0.06$; fig. 2A), while lymphocyte SOCS1 and CIS-1 in peritoneal dialysis and non-dialysis patients ($P = 0.06$, and $P < 0.01$ respectively; fig. 2B).

Table 1. General and biochemical data of controls and end stage renal disease (ESRD) subjects**.**

Variables	*Controls*	*ESRD*	*P*
N	15	30	-
Age (year)	52 ± 14	54 ± 19	0.64
F : M	8 : 7	15 : 15	-
BMI (kg/m^2)	25 ± 4	25 ± 5	0.76
GFR (1.73 / min / m^2)	80 ± 11	10 ± 4 (n=8)	<0.001
Systolic BP (mmHg)	121 ± 12	136 ± 21	<0.05
Diastolic BP (mmHg)	72 ± 6	82 ± 11	<0.01
Cholesterol (mmol/L)	5.6 ± 0.9	3.9 ± 1.0	<0.001
HDL (mmol/L)	1.4 ± 0.5	1.1 ± 0.3	0.08
LDL (mmol/L)	3.6 ± 0.7	2.0 ± 0.8	<0.001
Albumin (g/L)	45.1 ± 2.4	40.5 ± 3.7	<0.001
Glucose (mmol/L)	5.2 ± 0.5	5.3 ± 0.8	0.90
TG (mmol/L)	1.3 ± 0.5	1.8 ± 0.8	0.23
Hb (mmol/L)	9.4 ± 0.8	8.0 ± 1.0	<0.001
TBARS (µM)	3.6 ± 1.0	4.0 ± 1.9	0.35

Table 2. Medication used by end stage renal disease (ESRD) subjects.

Drug type	*Controls*	*ESRD*
Erythropoietin	None	23 of 30
Statins	None	17 of 30
ARB/ ACEi	None	23 of 30
Other anti-hypertensives	None	22 of 30
Anti-coagulants	None	11 of 30

Increased plasma levels of IL-6, TNFα and CRP

Plasma levels of IL-6 and TNFα, as well as CRP were significantly increased in end stage renal disease patients ($P < 0.001$, $P < 0.001$, and $P < 0.05$ respectively; fig. 3A). In contrast, plasma levels of IL-10 were not significantly changed (data not shown). IFNγ and IL-2 levels were below the lower detection limit of our method. Further analysis indicated that IL-6 was

significantly increased in non-dialysis, peritoneal dialysis, and hemodialysis patients ($P < 0.001$), TNFα in peritoneal dialysis and hemodialysis ($P < 0.001$) , and CRP in hemodialysis ($P < 0.001$) (fig. 3B). Correlation was found between monocyte SOCS1 and IL-6 in all end stage renal disease patients ($r = 0.43$, $P < 0.05$; fig. 4A), and between lymphocyte CIS-1 and TNFα in peritoneal dialysis patients only ($r = 0.73$, $P < 0.05$; fig. 4B). No other significant correlations were found between SOCS expression and the level of inflammatory marker.

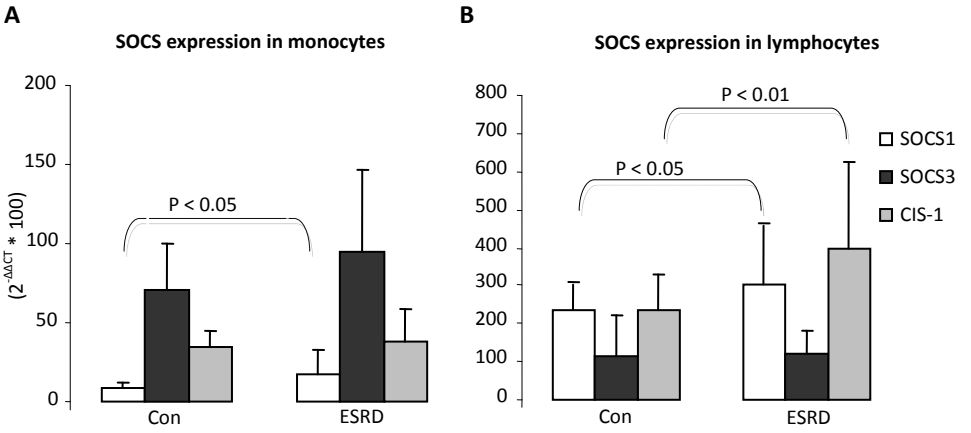


Fig. 1. Increased SOCS expression in monocytes and lymphocytes of end stage renal disease patients. SOCS1, SOC3 and CIS-1 gene expression in monocytes (A) and in lymphocytes (B) of healthy controls and end stage renal disease patients were measured using quantitative real time PCR as described under material and methods. The values are mean ($2^{-\Delta\Delta CT} * 100$) ± S.D. of gene expression in samples from 15 controls and 30 end stage renal disease subjects. RNA from lymphocytes of two controls and one end stage renal disease subjects and from monocytes of two controls were excluded because of RNA degradation. $P < 0.05$ was considered significant.

Discussion

Our study revealed increased monocyte SOCS1 and lymphocyte SOCS1 and CIS-1 in end stage renal disease patients, which was accompanied by increased plasma levels of IL-6, TNFα, and CRP, known biomarkers of inflammation and cardiovascular disease. Interestingly, monocyte SOCS1 correlated with plasma IL-6 levels, linking monocyte SOCS-1 to enhanced activity of this known marker of inflammation and cardiovascular disease. CIS-1 was significantly increased in lymphocytes of non-dialysis and peritoneal dialysis patients but not in hemodialysis, potentially pointing to different inflammatory conditions or cell responsiveness in hemodialysis patients (115), which could be related to the hemodialysis

procedure (116). For instance, hemodialysis membrane could increase inflammation by activating the mononuclear cells (117), though synthetic polysulfone membrane used in this study has higher biocompatibility and is more efficient in removing the oxidized low density lipoproteins (118, 119). Indeed CRP was only significantly increased in hemodialysis patients, but not in the other subgroups. We found a significant correlation between lymphocyte CIS-1 and TNFα, confirming a link between systemic inflammation and SOCS expression in mononuclear cells.

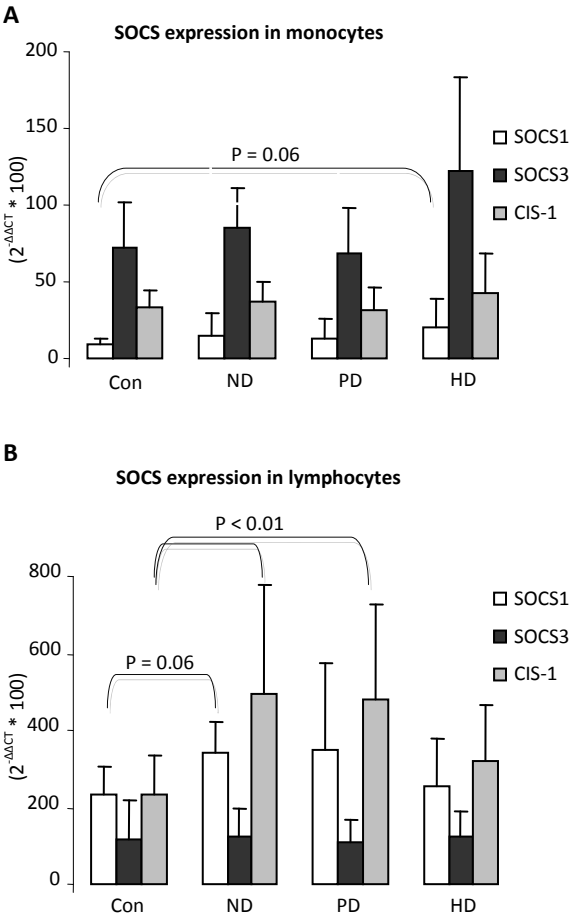


Fig. 2. Increased CIS-1 expression in lymphocytes of non-dialysis and peritoneal dialysis subjects. SOCS expression data of monocytes (A) and lymphocytes (B) of 8 non-dialysis, 8 peritoneal dialysis and 14 hemodialysis and 15 controls were compared to test the effect of dialysis and dialysis modality on SOCS expression in peripheral blood mononuclear cells (RNA from lymphocytes of two control and one non-dialysis subject, and monocytes of two control subjects were excluded because of RNA degradation). $P < 0.05$ was considered significant.

Increased SOCS expression is recognized in different inflammatory diseases (68). Likewise we report increased SOCS1 and CIS-1 expression in peripheral blood mononuclear

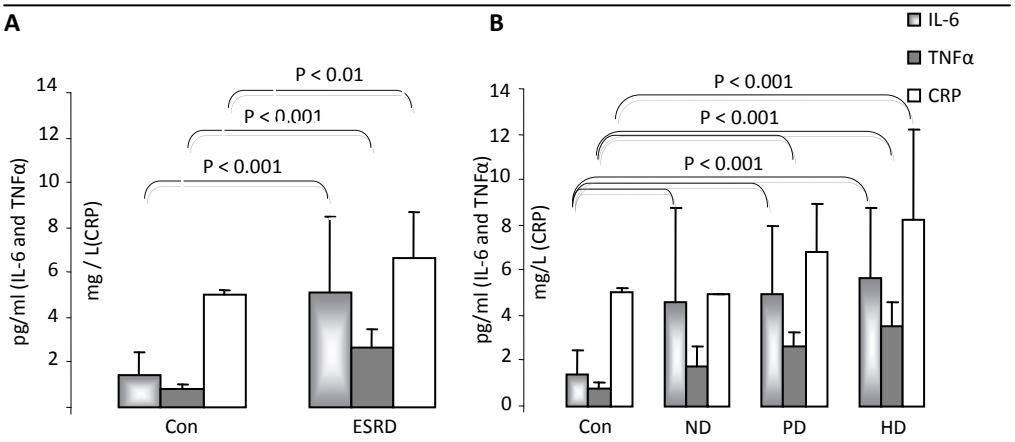


Fig. 3. Increased plasma levels of CRP and inflammatory cytokines in end stage renal disease patients. Plasma levels of IL-6, TNFα and CRP in healthy controls (n= 15) and end stage renal disease (ESRD) (n=30, IL-6 value of one hemodialysis patient was lost) (A) and in non-dialysis (ND; n=8), peritoneal dialysis (PD; n=8), hemodialysis (HD; n=14) and control (n=15) subjects (B) were measured as described in the material and methods section. The values are the mean ± S.D. n represents the number of subjects in each group.

cells of end stage renal disease patients, which could possibly reflect initiation or development of cardiovascular disease. SOCS1 and CIS-1 are important mediators of lymphocyte development and differentiation, and activation. SOCS1 is highly expressed in T helper-1 (Th-1) (109), while increased CIS-1 correlates with Th2 cell differentiation (30). Th-1 cells produce pro-inflammatory cytokines like IFNγ and TNFα; Th-2 cells produce anti-inflammatory cytokines like IL-10 (120) that antagonize Th-1 effects (120). Increased lymphocyte CIS-1 in non-dialysis and peritoneal dialysis might point to a predominance of Th2 cells (30, 109, 121). It has been suggested that in hemodialysis there is a shift to Th1 cells (23). However, we did not find increased lymphocyte SOCS1 in hemodialysis. That could be related to lymphocyte dysfunction in hemodialysis patients (122). Furthermore, insufficient SOCS1 expression in lymphocytes causes hyper-responsiveness of lymphocytes to different inflammatory cytokines (49). The expression of CIS-1 is selectively induced in T-cells after T-cell receptor stimulation (123), and prolongs survival of activated $CD4^+$ T-cells. Interestingly, the number of circulating $CD4^+$ is significantly higher in non-dialysis and peritoneal dialysis *versus* hemodialysis patients (124). The increased CIS-1 expression in lymphocytes of peritoneal dialysis and non-dialysis patients but not in hemodialysis, as shown here, could possibly reflect the lower number of $CD4^+$ cells in hemodialysis. Moreover, increase in circulating single positive T cells ($CD4^+$ and $CD8^+$) accompanied by a poor response to

erythropoietin (125), could be explained by CIS-1 being an intrinsic inhibitor of erythropoietin signaling (81).

In vivo and *in vitro* studies indicate a specific role for SOCS1 in inhibition of IFNγ signaling, which has a central role in atherosclerosis (112). However, studies in SOCS1 transgenic mice indicate that SOCS1 modulates a wider range of cytokine responses (126). Monocytes are involved in initiation and progression of atherosclerosis. Therefore, increased SOCS1 in monocytes, the precursors of macrophages, could delay monocyte responses to pro-inflammatory cytokines and delay atherosclerosis. In fact, SOCS1 inhibits IL-6-induced macrophage differentiation (127) and IFNγ-induced CD40 production that is involved in initiation of atherosclerosis (128). However, whether increased SOCS1 in monocytes of end stage renal disease patients is in proportion to increased pro-inflammatory cytokines and whether it could efficiently dampen pro-inflammatory signals remains unclear. Previous studies, however, indicate an increased number of activated monocytes with a pro-inflammatory phenotype in end stage renal disease (29, 129), which might suggest that increased monocyte SOCS1 is insufficient to dampen increased inflammatory pressure on monocytes. A similar phenomenon has been shown for example in synoviocytes of rheumatoid arthritis patients, which are hyper-responsive to IL-6, because of inadequate levels of SOCS3 (59).

Previously we reported increased monocyte SOCS3 in chronic kidney disease patients (15< GFR > 60 ml/min/1.73m^2) (113). SOCS3 expression in monocytes seems to have pro-atherogenic properties. This notion is based on a recent study that indicated decreased atherosclerosis in ApoE$^{-/-}$ mice, when SOCS3 was deleted from macrophages (62). Therefore, increased monocye SOCS3 in chronic kidney disease patients could be related to higher prevalence of cardiovascular disease. In fact, the chance of cardiovascular death in chronic kidney disease patients is 5-10 times more than the chance of reaching the end stage stadium (14). In the present study, we did not find significant changes in SOCS3 expression in end stage renal disease patients (all three subgroups). However, this is a cross-sectioned study and we did not follow the patients over the time. A subpopulation of patients with chronic kidney disease does not reach ESRD, since they die of cardiovascular disease. It can be speculated that this group has different monocyte SOCS3 expression in time than the patients

who survive and reach ESRD. Moreover, it could well be that advanced renal failure affects SOCS3 expression differently than milder chronic renal failure.

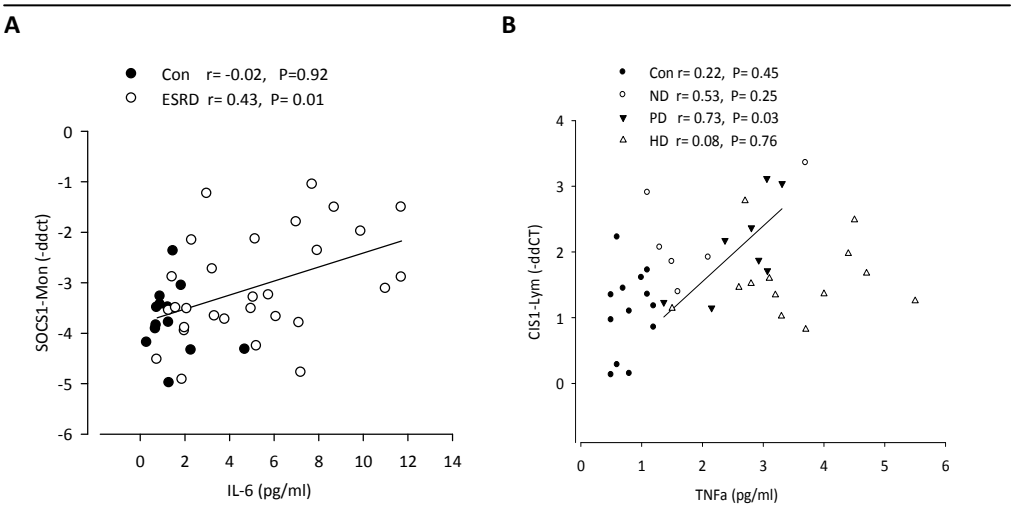


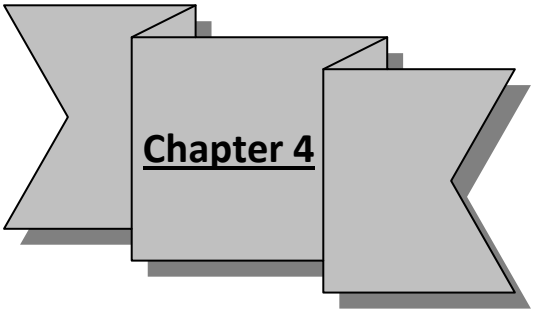
Fig. 4. Correlations between SOCS expression in peripheral blood mononuclear cells and inflammation. Correlations between monocyte SOCS1 and IL-6 in end stage renal disease (ESRD; n=29) (A), and lymphocyte CIS-1 and plasma TNFα in non-dialysis (ND; n=7), peritoneal dialysis (PD; n=8), hemodialysis (HD; n=14), and controls (n=13). RNA samples of one non-dialysis and two control subjects were excluded because of RNA degradation (B).

Theoretically medication could affect SOCS expression in peripheral blood mononuclear cells. For instance both erythropoietin and statins are able to induce SOCS in peripheral blood mononuclear cells (81, 105). However, resistance to erythropoietin therapy is frequent in end stage renal disease, and this is related to inflammation; possibly involving SOCS (130, 131). In accord, while all hemodialysis patients received erythropoietin, we did not find significant changes in SOCS expression in mononuclear cells of these patients. Furthermore, as we reported previously (113), our preliminary data in 3 chronic kidney disease patients indicated that medical intervention for four weeks did not affect SOCS expression in peripheral blood mononuclear cells significantly. Therefore, it is hard to define the involvement of medications in the changed SOCS expression in mononuclear cells of the patients in this study.

In conclusion, despite the relatively small number of subjects in this study we observed increased SOCS expression in peripheral blood mononuclear cells of end stage renal disease patients, indicating activation of intracellular inflammatory pathways. The SOCS profile in monocytes and lymphocytes was different, which appeared to be related to inflammation and dialysis modality. Unfortunately, we were unable to measure SOCS proteins in peripheral blood mononuclear cells because of technical difficulties. Hence, this should be addressed in follow up studies to confirm our data at the protein level.

Acknowledgments

We gratefully thank W.H. Boer, P.J. Blankestijn, P.F. Vos, N. Willekes and C. Tilburgs for their collaboration. This work was supported by the Dutch Kidney Foundation (NSN C03.2062) and the Genzyme Renal Innovations Program (GRIP).



SOCS3 a key component in negative cross talk between IFNγ and IL-6 signaling in endothelial cells

M. Mehdi Rastmanesh[¶], Branko Braam[¶†], Chantal Tilburgs[¶], Marie-Jose Goumans[§], Jaap Joles [¶], Peter Boer [¶], Hans A.R. Bluyssen[#]

[¶] Department of Nephrology and Hypertension, University Medical Center Utrecht, the Netherlands. [§] Department of Experimental Cardiology, University Medical Center Utrecht, the Netherlands.[†] Division of Nephrology and Immunology, Dept. Medicine, Univ. of Alberta, Edmonton, Canada and [4] Dept. Physiology, Univ. of Alberta, Edmonton, Canada. [#] Institute of Molecular Biology and Biotechnology, Adam Mickiewicz University, Poznan, Poland.

Submitted

Abstract

IL-6 has pro and anti-inflammatory effects. IL-6 signals through STAT3, which is in turn modulated by suppressors of cytokine signaling-3 (SOCS3). IL-6 is usually increased together with other inflammatory cytokines, that could affect IL-6 signaling and functions. We hypothesized that cross talk between IFNγ and IL-6 leads to increased inflammation in endothelial cells (EC), through inhibition of IL-6-induced STAT3 activation in a SOCS dependent manner.

Human microvascular endothelial cells (HMEC) stimulated with IL-6 in the presence or absence of IFNγ. STAT phosphorylation was measured by western blot analysis. Gene expression was studied using real time PCR. Gene expression was manipulated by actinomycin-D or RNA interference (RNAi) system.

IFNγ stimulation of HMEC cells resulted in STAT1 and 3 activation and SOCS1 and SOCS3 expression. IL-6 activated STAT3 and led to SOCS3 expression. IFNγ pretreatment of HMEC cells inhibited IL-6-inuced STAT3 activation, which was accompanied by increased SOCS3 protein. This effect did not occur when HEMC cells were co-stimulated with IFNγ and IL-6, when gene expression was inhibited using actinomycin-D or when SOCS3 expression was down regulated using siRNA. Furthermore, the cross-talk between IFNγ and IL-6 led to increased MCP-1.

Taken together, IFNγ inhibits IL-6-induced STAT3 activation in endothelial cells in a SOCS3 dependent manner, which differentially affects IL-6 downstream target genes. Increased MCP-1, a pro-atherogenic chemokine suggest that interaction between IFNγ and IL-6 is in favor of increased inflammation in EC.

Introduction

IL-6 is increased in patients with renal failure (95) as well as in patients with congestive heart failure (132). Plasma level of IL-6 is associated with progression of carotid atherosclerosis (133) and correlates with increased mortality and poor outcome in end stage renal disease patients (134, 135), and with mortality in the elderly (136). Furthermore, IL-6 serum reflects endothelial dysfunction, a characteristic of atherosclerosis development (137). Indeed, IL-6 promotes atherosclerosis in $ApoE^{-/-}$ mice (138). However, IL-6 deficiency enhances atherosclerotic plaque formation in $ApoE^{-/-}$ / $IL\text{-}6^{-/-}$ mice (139), and endogenous IL-6 diminishes inflammatory response by controlling the level of pro-inflammatory cytokines (140, 141). Together these data indicate both pro- and anti-inflammatory as well as pro- and anti-atherogenic effects of IL-6.

IL-6 is commonly induced together with other inflammatory cytokines that could affect IL-6 signaling and its biological effects. In accord, IL-1β, TNFα, IL-10, and LPS interfere with IL-6 signaling, and diminish its anti-inflammatory effects (63, 142). Therefore, the function of IL-6 as a pro or anti-inflammatory cytokine is also related to the presence or absence of other inflammatory cytokines.

IFNγ is a pro-inflammatory cytokine with a central role in atherosclerosis (112). Strikingly, IFNγ and IL-6 share the same intracellular signaling molecules to induce a partially overlapping set of genes (143), though they have opposing biological effects. At signaling level, upon binding to their cognate receptors, IFNγ and IL-6 activate Jak1, 2 and Tyk2 from Jak family of kinases (144, 145), which terminate in activation of STAT1 and 3 in different cell types (146, 147). However, STAT1 is the main transcription factor in IFNγ signaling (148) and STAT3 in IL-6 signaling (149). Interestingly, these two transcription factors have opposing actions, despite structural homologies (150, 151). For instance, while STAT1 has apoptotic action, STAT3 inhibits apoptosis (150). This could explain differences in IFNγ and IL-6 functions. However, in the absence of STAT3, IL-6 signals through STAT1 and induces IFNγ-like signals (152). This suggests that activation of STAT3 upon IL-6 signaling is the key element to define to what extent IL-6 functions differ from IFNγ.

The next important difference in IFNγ and IL-6 signaling is related to how their signaling pathway is being modulated. In general, activation of Jak/STAT pathway is mainly modulated through inducible proteins of suppressors of cytokine signaling (SOCS). In vivo studies indicate a selective role for SOCS1 in inhibition of IFNγ (108) and of SOCS3 in IL-6 signaling (101). Moreover, in the absence of SOCS3, IL-6 has anti-inflammatory effects in macrophages, which is related to the prolonged IL-6-induced STAT3 activation (153). As anti-inflammatory effects of IL-6 rely on STAT3 activation, molecules that induce SOCS expression could potentially affect IL-6 signaling and its physiological effects. In accord, LPS, IL-10, and TNFα inhibit IL-6-induced STAT3 activation that is accompanied by increased SOCS3 expression (63, 142). Therefore, the physiological role of IL-6 is closely related to the presence of other factors that induce SOCS expression. IFNγ induces SOCS in many cell types including endothelial cells, however, it is unclear whether IFNγ could interfere with IL-6 action, whether the interference is related to SOCS, and how this affect IL-6 signaling in endothelial cells. Therefore, in the present study we hypothesized that cross talk between IFNγ and IL-6 in endothelial cells leads to increased inflammation (pro-atherogenic effect) through inhibition of IL-6-induced STAT3 activation in a SOCS dependent manner. This could present a common phenomenon in how inflammation shifts the IL-6 signaling in favor of inflammation.

Material and methods

Reagents and cell culture

Human Micro-vascular Endothelial Cells were generously provided by Centers for Disease Control and Prevention (Atlanta, USA). Recombinant IFNγ and IL-6 were purchased from Sigma. Rabbit polyclonal antibodies P-Tyr (701) STAT1, and mouse antibody to P-Tyr (705) STAT3, goat polyclonal antibody to SOCS3, and HRP conjugated antibodies were obtained from Santa Cruz Biotechnology. Mouse monoclonal antibodies to STAT1, and STAT3 were purchased from BD Biosciences. Generally, one day before an experiment, the MCDB-131 (Invitrogen) medium supplemented with penicillin/streptomycin 1%, hEGF 0.1%, hydrocortisone 0.1%, L-glutamine 5%, and 10% fetal calf serum (Invitrogen) was replaced

with MCDB-131 containing 0.5% FCS and penicillin/streptomycin 1%. However, in RNAi (siRNA) experiments HMEC cells that were transfected with RNAi or not received serum free MCDB-131, 1 hour before cytokine stimulation. RNAi against SOCS1, SOCS3 and GAPDH and off-target siRNA were purchased from Dharmacon.

Gene expression assay

After total RNA was isolated using TRIzol® (Gibco), 1 μg RNA was reverse transcribed (iScript® cDNA synthesis Kit, Bio-RAD). Gene expression was assessed by real-time PCR (Applied Biosystems TaqMan® Assays-on-Demand™ Gene Expression Products) using pre-designed SOCS1, SOCS3, MCP-1, GAPDH and 18S primers on the Applied Biosystems, 7900 HT Fast Real-Time PCR System. The amount of target gene in each subject was normalized to endogenous controls 18S (ΔCT). Gene expression is expressed as mean ($2^{-\Delta CT}$ *100) ± S.D. of at least six independent experiments.

Western analysis

Total cell extracts (15 μg protein for STAT or 50 μg for SOCS), from untreated and treated HMEC were electrophoresed on a 4-20 % pre-cast acrylamide gradient gel (Cambrex) or 10% acrylamide gel (for SOCS) and transferred to nitrocellulose membrane (Roche). After blocking in ECL-blocking buffer the membrane was either incubated with polyclonal antibodies for P-Tyr STAT1 and 3 (1:1000), or SOCS3 (1/500). After 3 times washing with PBS-Tween (PBST) the membrane was incubated with goat anti-mouse, goat anti-rabbit or rabbit anti-goat HRP conjugated (1: 1000) antibody. Next, the membrane was washed 2 times with PBST and once with PBS and bound proteins were detected with enhanced chemoluminescence (ECL, Amersham). The membrane was then stripped, blocked and incubated with specific monoclonal antibodies for STAT1, STAT3 (1:5000), to measure the total amount of STAT proteins. The intensity of each band was quantified using Quantity One program (BioRad) and normalized on the level of STAT proteins. Results are expressed as mean (P-STAT/STAT or SOCS3/ STAT3) ± S.D. of at least six independent experiments.

Small Interference RNA (siRNA)

HMEC cells were plated on fibronectin coated 6 well plates (5 * 10^5 cells/well) and incubated at 37°C with 5% CO_2. Sub-confluent cells were transfected with siRNA (50nM) using DharmaFECT1 (2 µl/ml) (Dharmacon) in serum and antibiotics free medium. Transfection efficiency was tested and optimized using Cy3 probe (data not shown). siRNA against GAPDH was used as a positive control and to optimize the system (data not shown). Off target effect was tested using a mix of 4 unmatched sequences of siRNA (data not shown). HMEC cells were stimulated with IFNγ (1ng/ml) or IL-6 (50 U/ml) 48 hr post-transfection..

Experimental design

1. Time course study of IFNγ and IL-6 were performed by stimulation of HMEC with IFNγ (1ng/ml) or IL-6 (20 U/ml) for 0-6 hours. Cell lysates and RNA were isolated to study the activation of STAT1, and STAT3 or the levels of SOCS proteins and the expression of SOCS1, and SOCS3 genes.
2. Concentration course study of IFNγ (data not shown) and IL-6 were performed by stimulation of HMEC with 0-5 ng/ml IFNγ or 0-50 U/ml IL-6 for 30 and 120 minutes. Cell lysates and RNA were isolated to study the activation of STAT1, and STAT3, or the levels of SOCS proteins (at 30 min) or the expression of SOCS1, and SOCS3 genes (at 2 h).
3. The effect of IFNγ on IL-6 signaling was studied by incubation of HMEC with IFNγ (1ng/ml) 2 hours prior to IL-6 stimulation for 30 minutes (for STAT activation) or 60 minutes (for gene expression).
4. The role of SOCS in cross-talk between IFNγ and IL-6 was determined by: a) co-stimulation of HMEC with IFNγ (1ng/ml) and IL-6 (50 U/ml) for 30 minutes; b) by pretreatment of HMEC cells with actinomycin-D (Act-D; 10 µg/ml) for 15 min prior to stimulation with IFNγ (1ng/ml; 2h) and IL-6 (50 U/ml; 30 min), and; c) down-regulating SOCS3 or SOCS1 and SOCS3 by siRNA prior (48 hours) to stimulation with IFNγ (1ng/ml; 2h) and IL-6 (50 U/ml; 30 min).

Statistical analysis

Results are expressed as mean ± SD. Data were compared by a One Way ANOVA and the studentized Newman Keuls post-hoc test, when appropriate. If data were not distributed normally we used Kruskal-Wallis ANOVA on ranks and Dunn's post-hoc test. All statistical tests were performed with SigmaStat (version 3.5, Systat Software Inc, San Jose, Ca); $p < 0.05$ was considered to be significant.

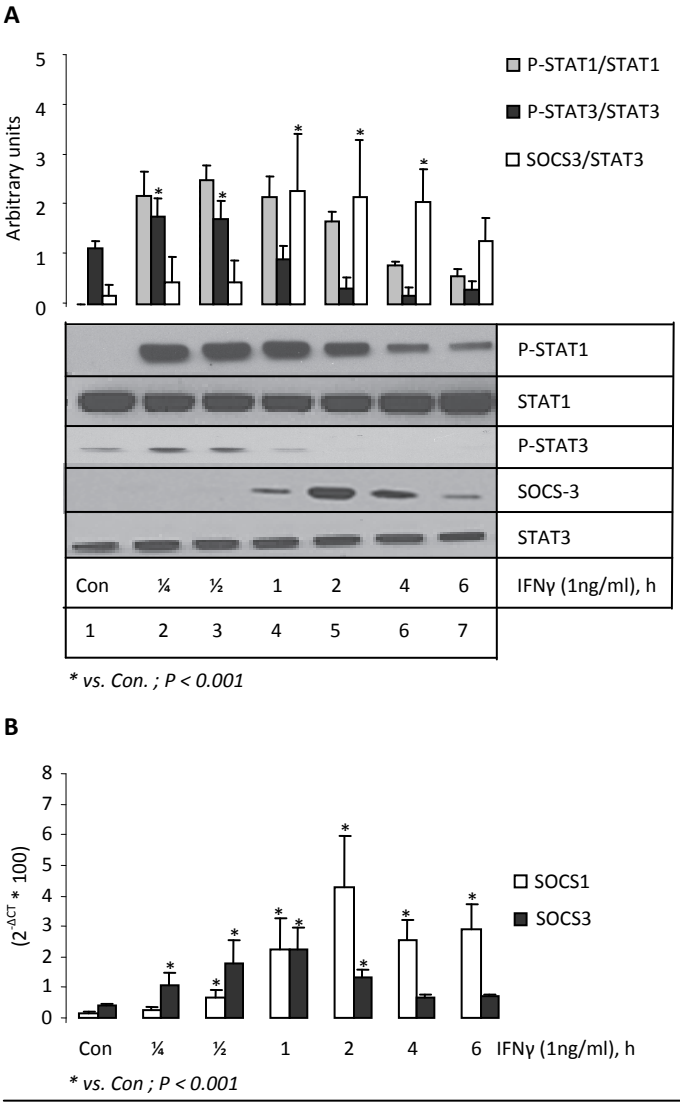


Fig. 1. IFNγ activated STAT1 and STAT3 and induces SOCS1 and SOCS3 in EC. HMEC cells were treated with IFNγ (1 ng/ml) **(A-B)** for up to 6 hours. **(A)** Cell lysates were analyzed by western blot for activated STAT and SOCS protein levels. Data were analyzed by Kruskal-Wallis Anova On Ranks / Dunn's method. **(B)** SOCS expression was assessed using real time PCR and normalized on 18S expression. Data were analyzed by Kruskal-Wallis Anova On Ranks / Dunn's method. P < 0.05 considered significant.

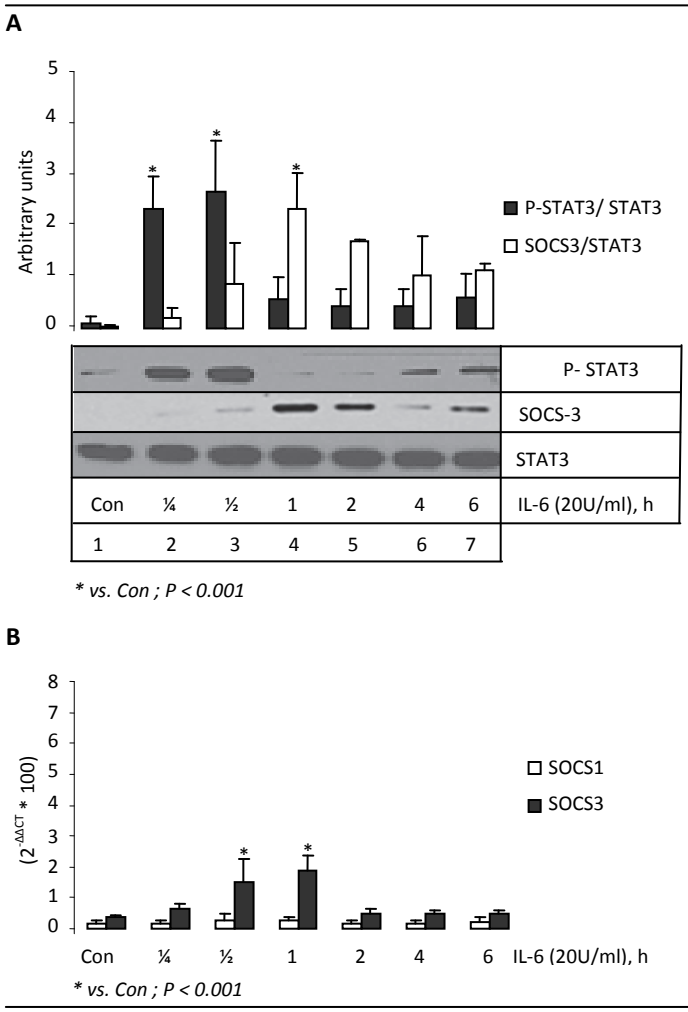


Fig. 2. IL-6 activated STAT3 and induces SOCS3 in EC. HMEC cells were treated with IL-6 **(A-B)** for up to 6 hours. **(A)** Cell lysates were analyzed by western blot for activated STAT and SOCS protein levels. Data were analyzed by Kruskal-Wallis Anova On Ranks / Dunn's method. **(B)** SOCS expression was assessed using real time PCR and normalized on 18S expression. Data were analyzed by Kruskal-Wallis Anova On Ranks / Dunn's method. P < 0.05 considered significant.

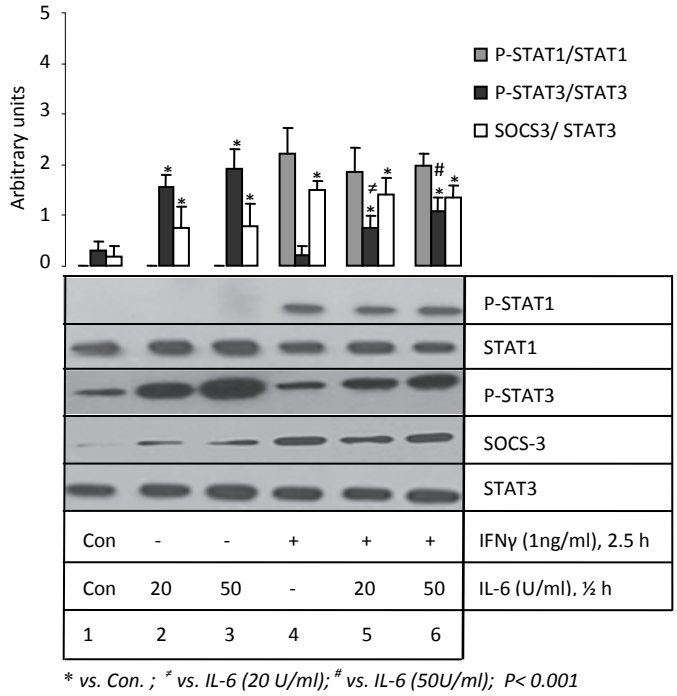
Results

IFNγ activated STAT1 and STAT3 and induced SOCS1 and SOCS3 expression in EC in a time-dependent fashion.

In a time-dependent manner IFNγ activated both STAT1 and STAT3 in EC (fig. 1A). STAT1 activation was already significant after 15 minutes with a concentration of IFNγ as low as 1 ng/ml that reached its peak at 30 min. STAT1 activation decreased after one hour, but it was still detectable even after 6 hours. STAT3 activation was, however, weak and short-lived and decreased to the normal level after one hour. This was probably due to increased SOCS3

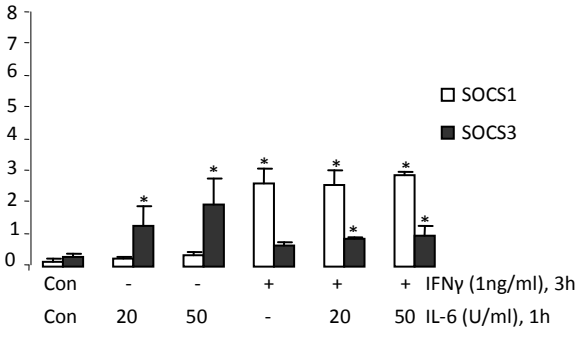
protein, which was significantly increased after one hour and started to decrease after 4hours. IFNγ-induced STAT activation was not increased at higher concentrations (data not shown).

A



** vs. Con. ; ≠ vs. IL-6 (20 U/ml); # vs. IL-6 (50U/ml); P< 0.001*

B



** vs. Con. ; P < 0.001*

Fig. 3. IFNγ pretreatment of EC inhibited IL-6 induced STAT3 activation and SOCS3 expression. HMEC cells were pretreated with IFNγ (0-1ng/ml) 2 hours before IL-6 stimulation (0-50 U/ml) for another 30 minutes. (**A**) Cell lysates were prepared to study the STAT activation and SOCS protein levels. Data were analyzed by One Way Anova / Student-Newman-Keuls method. **(B)** IL-6 stimulation took 1 hour post IFNγ pretreatment of EC. mRNA were prepared to study SOCS expression using real time PCR. Data were analyzed by Kruskal-Wallis Anova On Ranks / Dunn's method. P< 0.05 considered significant.

STAT activation was followed by increased expression of SOCS1 and SOCS3 mRNAs (fig. 1B). SOCS1 was maximally induced after 2 hours and it was still significantly present after 6 hours (even at later time-points; data not shown). Similar to STAT3 activation, SOCS3 gene induction by IFNγ was much weaker than SOCS1 and it was short-lived. However, SOCS3 induction was stronger than SOCS1 at earlier time points. SOCS3 gene expression preceded the increased SOCS3 protein (compare fig. 1B with fig. 1A), the latter was accompanied by decreased STAT3 activation (fig. 1A, lanes 4-7).

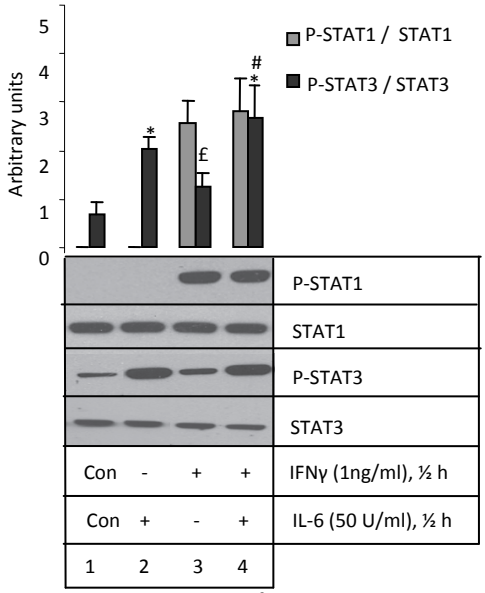
IL-6 activated STAT3 and induced SOCS3 expression in EC in a time- and concentration-dependent fashion.

In contrast to IFNγ, IL-6 activated only STAT3 in EC (fig. 2A). IL-6-induced STAT3 activation had a peak at 30 minutes and rapidly decreased at later time points. This was accompanied by increased SOCS3 protein at 30 min, which reached its peak at 1 hour. At later time points (after 4 hours) SOCS3 protein decreased, and that was followed by a slight increase in STAT3 activation. Increased SOCS3 protein level was preceded by increased SOCS3 gene expression upon IL-6 stimulation, which was maximally increased within 30-60 minutes (fig. 2B).

IFNγ inhibited IL-6-induced STAT3 activation and SOCS3 expression.

Cross-talk between IFNγ and IL-6 signaling was studied by incubation of EC with a low concentration of IFNγ (enable to induce SOCS1 and SOCS3 (gene and protein); fig. 1 and 2) prior to IL-6 treatment. Pre-treatment of EC with IFNγ significantly decreased IL-6-induced STAT3 activation (fig. 3A, compare lanes 2-3 with 5-6), that was accompanied by increased IFNγ-induced SOCS3 protein level (fig. 3A, lanes 4-6). Similar to STAT3 activation, IL-6-induced SOCS3 gene expression was decreased (fig. 3B) by pretreatment of EC with IFNγ. This interaction, however, did not affect STAT1 activation or SOCS1 gene expression (fig. 3A-B).

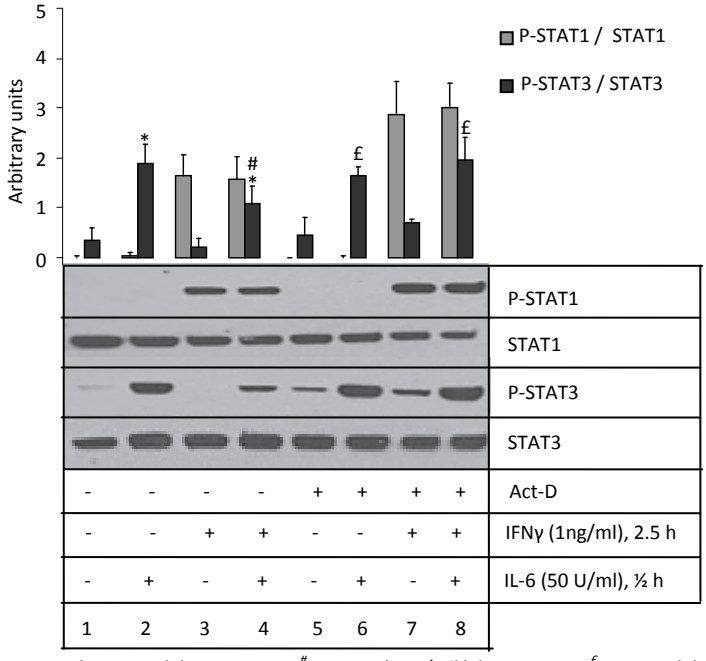
A



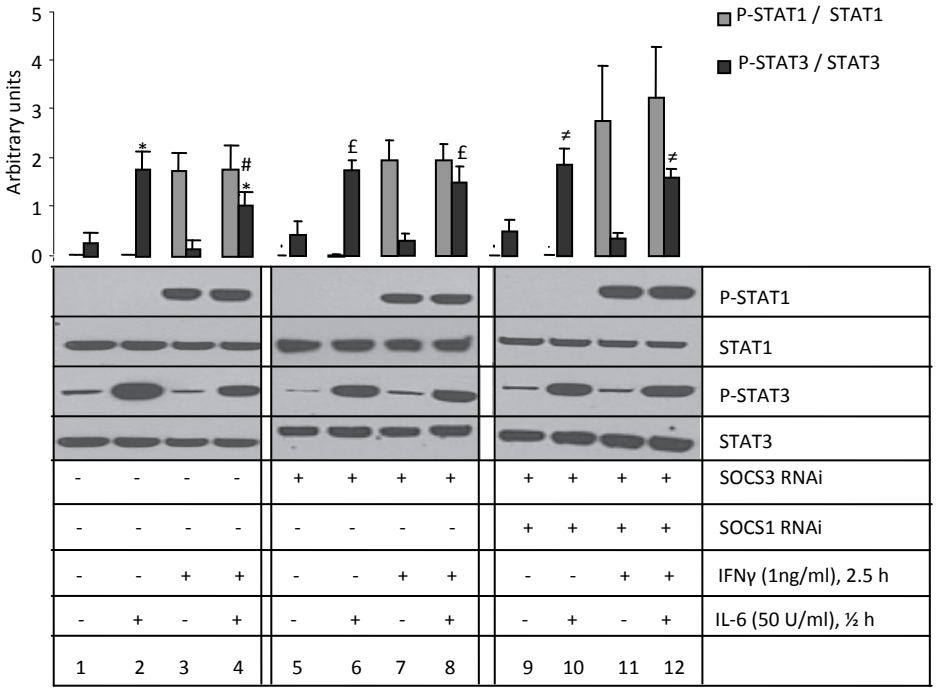
** vs. Con. ; P < 0.001 , £ vs. Con; P< 0.05*
 # vs. IL-6 (50U/ml); P< 0.01

Fig. 4. Inhibitory effect of IFNγ on IL-6 signaling is dependent on de novo genes/proteins induction. HMEC cells were treated with IFNγ (0-1ng/ml) and IL-6 (0-50 U/ml) simultaneously for 30 min **(A),** or incubated with actinomycine-D (10 µg/ml) for 15 min (**B**) prior to IFNγ (0-1ng/ml) stimulation for 2 hours and IL-6 (0-50 U/ml) for additional 30 minutes**.** Cell lystes were prepared to study STAT activation by western blot analysis. Data were analyzed by One Way Anova / Student-Newman-Keuls method (**A**, and **B** bands1-4), or by Kruskal-Wallis Anova On Ranks / Dunn's method (**B**; Bands 5-8). P< 0.05 considered significant.

B



** vs. Con. (1). ; P < 0.001, # vs. IL-6 (50U/ml)(2) ; P< 0.001, £ vs. Con. (5); P< 0. 01*



** vs. Con. (1). ; P < 0.001, # vs. IL-6 (50U/ml)(2) ; P< 0.01,*
£ vs. Con. (5); P< 0.001, ≠ vs. Con. (9); P< 0.001

Fig. 5. Inhibitory effect of IFNγ on IL-6 signaling is SOCS3-dependent. HMEC cells incubated with SOCS3 RNAi (bands 5-8) or SOCS1 and SOCS3 RNAi (bands 9-12) for 48 hours prior to IFNγ (0-1ng/ml) stimulation for 2 hours and IL-6 (0-50 U/ml) for additional 30 minutes. Cell lystes were prepared to study STAT activation by western blot analysis. Data were analyzed by One Way Anova / Student-Newman-Keuls method. P< 0.05 considered significant.

Inhibition of IL-6-induced STAT3 activation by IFNγ is SOCS-dependent

To test whether the effect of IFNγ on IL-6-induced STAT3 activation was related to de novo gene or protein induction, EC were co-stimulated with IFNγ and IL-6 for 30 minutes. Interestingly, this approach totally eliminated the inhibitory effect of IFNγ on IL-6-induced STAT3 activation (fig. 4A, compare lane 2 with 4). Furthermore, incubation of EC with actinomycin-D that inhibits genetranscription, totally reversed the inhibitory effect of IFNγ on IL-6-induced STAT3 activation (fig. 4B; compare lanes 2 with 4, and 6 with 8).

We suggested that elevated SOCS expression by IFNγ could inhibit IL-6-induced STAT3 activation. To test this hypothesis, SOCS1 (data not shown), SOCS3 or both SOCS1 and SOCS3 were knocked down in EC using siRNA method. While downregulation of SOCS3 almost

reversed the inhibitory effect of IFNγ on IL-6-induced STAT3 activation, downregulation of SOCS1 did not have a major effect. This is also indicated when both SOCS1 and SOCS3 were downregulated , which did not further increase STAT3 activation (fig. 5, compare lane 2 with 4, 6 with 8, and 10 with 12).

Cross-talk between IFNγ and IL-6 increased MCP-1 expression in EC

Although, IFNγ pre-treatment of EC resulted in decreased IL-6-induced STAT3 activation and SOCS3 gene expression, it did not decrease MCP-1 expression (fig. 6). IFNγ and IL-6 both are able to induce MCP-1 expression in EC, though not significantly. However, pre-treatment of EC with IFNγ, resulted in significant increased MCP-1 expression upon IL-6 stimulation (fig. 6).

Discussion

Like many other cytokines, IL-6 signals through Jak/STAT pathway, and its signal is being mainly modulated through SOCS proteins (63). It is, therefore, conceivable that possible cross-talk between IL-6 and other cytokines through Jak/STAT and modulating SOCS proteins could be of paramount importance in IL-6 signaling and its biological effects. Indeed, previous studies have demonstrated the inhibitory effects of IL-10, LPS, TNFα, and IL-6 on IL-6-induced STAT3 activation along with increased SOCS3 (63, 142). SOCS3 is in particular an interesting protein in cross-talk between IL-6 and other inflammatory cytokines. This notion is based on the *in vivo* observation that SOCS3 specifically inhibits IL-6 signaling (101), and also because it modulates not only the quantity of IL-6 signaling but also it affects the quality of IL-6 signaling (153). Yasukawa et al (153) demonstrated that SOCS3 diminishes the anti-inflammatory effects of IL-6 by inhibiting the IL-6-induced STAT3 activation, indicating that anti-inflammatory effects of IL-6 rely on STAT3 activation (63). Therefore, we suggested that under inflammatory conditions (such as uremia), possible increment of SOCS expression in EC could inhibit IL-6-induced STAT3 activation and consequently its anti-inflammatory effects. To test this idea we studied the cross-talk between IFNγ and IL-6. We hypothesized that cross-talk between IFNγ and IL-6 in endothelial cells leads to increased inflammation (pro-atherogenic effect) through inhibition of IL-6-induced STAT3 activation in a SOCS dependent

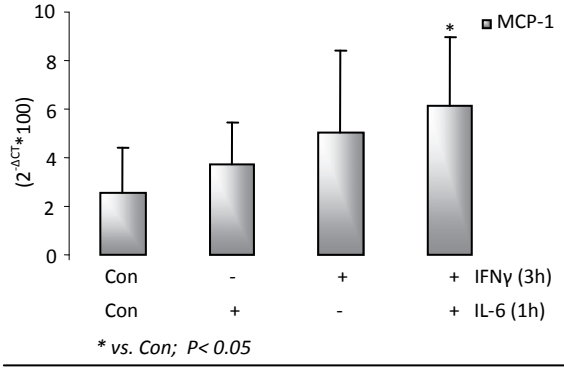


Fig. 6. Cross talk between IFNγ and IL-6 in EC leads to increased MCP-1 expression. HMEC cells were stimulated with IFNγ and IL-6 as described under fig. **3B**. MCP-1 gene expression was assessed using real time PCR. Data were analyzed by Kruskal-Wallis Anova On Ranks / Dunn's method. P< 0.05 considered significant.

manner. This could present a common phenomenon regarding how inflammation may shift the IL-6 signaling in favor of inflammation.

IFNγ was a very interesting candidate. This is based on the fact that IFNγ is increased in atherosclerotic plaques and has a central role in atherosclerosis development (112). It uses the same components of Jak/STAT pathway as does IL-6, to induce partially overlapping sets of genes including SOCS1 and SOCS3 in different cell types (143, 154). Furthermore, a single switch from STAT3 to STAT1 makes IL-6 have IFNγ-like or pro-inflammatory and pro-atherogenic effects (152). Therefore, IFNγ could also function as a positive control to evaluate to what extent a possible cross-talk between IFNγ and IL-6 could increase the pro-inflammatory effects of IL-6.

We observed that IFNγ activates both STAT1 and STAT3 in EC. However, while STAT1 activation was long lasting, STAT3 activation was short-lived. This latter was accompanied by increased SOCS3 protein levels. IFNγ also induced SOCS1 and SOCS3 gene expression in EC. Similarly, SOCS1 expression was long lasting, while SOCS3 expression was transient. Time course study of IFNγ-stimulated EC indicated that IFNγ-induced SOCS3 gene expression preceded the increased SOCS3 protein level (unfortunately, because of technical difficulties we were not able to detect SOCS1 protein), which led to fast inhibition of IFNγ-induced STAT3 activation. This suggests a novel mechanism through which early induction of SOCS3 steers IFNγ to signals through STAT1, by blocking the STAT3 pathway. This also strongly suggests that SOCS3 is able to bind to IFNγ receptor as well. In contrast to IFNγ, IL-6 solely activated STAT3, and led to predominant SOCS3 (gene and protein) expression and slight induction of SOCS1 mRNA at higher concentrations. However, similar to IFNγ and similar to IL-6 signaling

in macrophages, IL-6-induced STAT3 activation was transient, that could be explained by the fast induction of SOCS3 and its inhibitory effect on IL-6-induced STAT3 activation (63). Interestingly, the kinetics of STAT3 activation and SOCS3 (gene and protein) expression upon IFNγ or IL-6 stimulation of EC are similar. This suggests a unique pattern of STAT3 activation and SOCS3 expression regardless the stimulus that leads to STAT3 activation and SOCS3 induction.

IFNγ-induced SOCS expression in EC created a desirable condition for our intension to study how increased SOCS might affect IL-6 signaling. Indeed, we observed that IFNγ pre-treatment of EC caused inhibition of IL-6-induced STAT3 activation, and decreased SOCS3 expression upon IL-6 stimulation. These data were so far in line with other studies, in which other inflammatory cytokines inhibited IL-6-induced STAT3 along with increased SOCS3 expression (63, 142). Our data also indicated that inhibitory effects of IFNγ on IL-6-induced STAT3 activation were accompanied by increased SOCS1 and SOCS3 gene expression (and SOCS3 protein level). However, the question remained unanswered whether SOCS is indeed functionally involved in this cross-talk and if so which SOCS is the important one. For instance, LPS induces SOCS3 expression in macrophages, but its inhibitory effect on IL-6-induced STAT3 activation occurs rapidly and does not depend on de novo gene transcription (63). Furthermore, phorbol 12-myristate 13-acetate (PMA) and ionomycin also inhibit IL-6-induced STAT3 activation. These inhibitory effects are also rapid and do not require new RNA or protein synthesis (155). In our study the inhibitory effect of IFNγ was, however, related to de novo protein induction. This is because co-stimulation of EC with IFNγ and IL-6 (which would not allow de novo protein induction prior to IL-6 stimulation (63)) did not show the inhibitory effect on IL-6-induced STAT3 activation. Furthermore, inhibition of de novo gene and protein induction by Act-D (63), completely reversed the inhibitory effect of IFNγ on IL-6-induced STAT3 activation, confirming the necessity of de novo gene and protein induction in cross-talk between these two cytokines. At last, to confirm that this phenomenon was related to SOCS proteins and not to other possible inducible genes, SOCS1 and SOCS3 mRNA were down-regulated using RNAi system. This experiment nicely indicated that down-regulated SOCS partially reversed the inhibitory effect of IFNγ on IL-6-induced STAT3 activation, and in this regard, SOCS3 plays the central role.

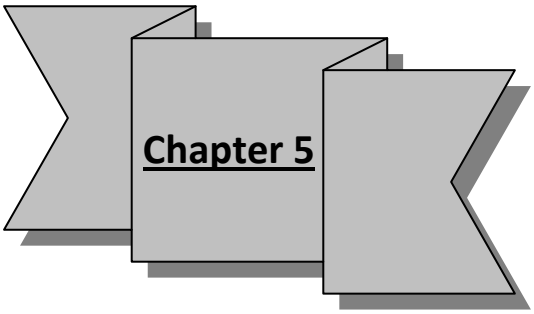
As anti-inflammatory effects of IL-6 is related to STAT3 activation, cross-talk between IFNγ and IL-6 could inhibit the anti-inflammatory effects of IL-6. It is known that in the absence of STAT3, IL-6 might have IFNγ-like effects (152). It was therefore, interesting to know whether inhibition of IL-6-induced STAT3 activation could somehow increase the pro-inflammatory or IFNγ-like effects of IL-6. Interestingly, pre-treatment of EC with IFNγ, led to an additive effect of IFNγ and IL-6 on MCP-1 expression in EC. MCP-1 is a chemokine with atherogenic properties. MCP-1 enhances atherosclerotic plaques formation by mediating monocyte/macrophage infiltration into arterial wall and foam cell formation (156). IFNγ induces MCP-1 in a STAT1-independent fashion(157). Our study indicated that Inhibited IL-6-induced STAT3 activation did not lead to decreased IL-6-induced MCP-1 expression, suggesting that MCP-1 expression is also STAT3-independent. These data together suggest that MCP-1 expression should be triggered by other transcription factor(s) rather than STAT1 or STAT3.

Together, our data indicate that the inhibitory effect of IFNγ on IL-6-induced STAT3 activation, differentially affects the expression of IL-6 target genes. This is in accord with a previous report in which IL-10 treatment of monocytes inhibited the IFNγ-induced ICAM-1 and the chemokine inducible protein-10 (IP-10) but not the IFNγ induction of interferon regulatory factor-1 (IRF-1) (54).

In sum, IFNγ pretreatment of EC inhibits IL-6 induced STAT3 activation and differentially modulate the expression of IL-6 downstream genes. It is tempting to speculate that a part of IL-6 signaling pathway is inhibited by IFNγ pretreatment of EC, which suppresses anti-inflammatory effects, but does not affect (or stimulates) the pro-inflammatory responses. Therefore, The outcome of inflammation is not only dependent on the level of pro and anti-inflammatory cytokines (158), but also on how cytokines affect each others signaling.

Acknowledgements

We gratefully thank Centers for Disease Control and Prevention (Atlanta, USA) for providing the HMEC cells. We also thank Dr. L.H. Ulfman for her technical advices.



Modulation of the IL6-induced transcriptome by IFNγ in microvascular endothelial cells in culture

Mehdi Rastmanesh[1], Hans Bluyssen[2], Kim Jie[1], Jaap. A. Joles[1], Sebastiaan Wesseling[1] and Branko Braam[1,3,4,]

[1] Department of Nephrology and Hypertension, University Medical Center Utrecht, the Netherlands. [2] Laboratory of Human Molecular Genetics, Institute of Molecular Biology and Biotechnology, Faculty of Biology, Adam Mickiewicz University, Poznan, Poland. [3] Division of Nephrology and Immunology, Dept. Medicine, Univ. of Alberta, Edmonton, Canada and, [4] Dept. Physiology, Univ. of Alberta, Edmonton, Canada.

In preparation

Abstract

IL-6 has both pro and anti-inflammatory effects. IL-6 is mostly increased under inflammatory conditions along with other inflammatory cytokines that could affect IL-6 signaling and function. Previously we reported that IFNγ-pretreatment of EC inhibits IL-6 induced SOCS3 and increased IL-6-induced MCP-1 expression. In the present study we tested the hypothesis that part of the pro-inflammatory response of IL-6 would be enhanced by IFNγ and part of the anti-inflammatory response inhibited.

Transcriptional responses of IL-6 in the presence or absence of IFNγ as well as the transcriptome of IFNγ in EC were studied using Illumina ® microarrays. Of several key genes the microarray gene expression data were confirmed by real time PCR.

Our data indicates that IL-6 induces a limited number of genes in EC with both pro and anti-inflammatory properties including SOCS3, MCP-1, Id-1 and Myc. IFNγ induced a number of chemokines and antproliferative genes including IP-10, GBP1, and IRF1. IFNγ-pretreatment of EC lowered the expression of SOCS3, Id-1, and Myc, while the expression of other genes including chemokines MCP-1, CXCL11, and CCL8 was increased. NLF2 is a new identified target of both IFNγ and IL-6 in EC.

This is the first report of IL-6 induced transcriptome in EC. Besides the known IL-6 target genes (e.g. SOCS3, Myc, and Id-1) we identified NLF2 as a new target of IL-6 (and also of IFNγ) in EC. IFNγ-pretreatment of EC lowered the expression of some proliferative and protective target genes of IL-6 such as Id-1 and Myc. IL-6 and IFNγ stimulation of EC had an additive effect on the expression of NLF2, and MCP-1. Furthermore, IFNγ increased adhesiveness of EC through induction of genes involved in adhesion including CCL8, CXCL11, and CXCL2. The expression of these genes was increased in the presence of IL-6 that might present a new mechanism involved in EC dysfunction and consequently increased atherosclerosis.

Introduction

Endothelial cells (EC) cover the vessel wall and play critical roles in controlling vascular tone and adhesion of circulating (mononuclear) cells, and inflammatory responses (159). The function of EC is affected by systemic inflammation that leads to production of cell surface adhesion molecules, chemokines and inflammatory cytokines, which are characteristics of EC dysfunction (160). The latter is involved in initiating of atherosclerosis lesions (33).

IL-6 is increased under different inflammatory conditions including chronic renal failure (95, 132, 161). Increased plasma IL-6 levels are demonstrated to be related to EC dysfunction and atherosclerosis development in renal patients (33). However, IL-6 is a pleiotropic cytokine with both pro- and anti-inflammatory properties (95, 140). Whether IL-6 affects EC function as a protective or damaging cytokine is still far from clear. IL-6 uses the same components of Jak/STAT pathway as the pro-inflammatory cytokine IFNγ and the anti-inflammatory cytokine IL-10. In fact IL-6 may have both IFNγ-like and IL-10-like effects under certain circumstances (63, 143, 152). For instance, similar to IL-10, IL-6 inhibits cytokine production by activated macrophages, which is related to IL-6-induced STAT3 activation (63). This latter is the main transcription factor in IL-6 and IL-10 signaling cascade (63). Nevertheless, the anti-inflammatory effect of IL-6 is supposedly weak, which is related to the high sensitivity of IL-6-induced STAT3 activation to the inhibitory effect of inducible suppressor of cytokine signaling-3 (SOCS3) (63). Moreover, in STAT3 knock out fibroblast cells, IL-6 signals through STAT1 (the main transcription factor in IFNγ signaling cascade), and has IFNγ-like effects (152). These data indicate that STAT3 activation is the key element that makes IL-6 signaling different from IFNγ. Simultaneously, higher sensitivity of IL-6-induced STAT3 activation to the inhibitory effect of SOCS3 makes it different from IL-10.

IL-6 is frequently induced along with other inflammatory cytokines that could affect IL-6 signaling and actions. For instance, IL-6 is increased together with IFNγ in atherosclerosis lesions (162). Previously, we reported that IFNγ pretreatment of EC results in inhibition of IL-6-induced STAT3 activation in a SOCS-dependent manner (chapter 4). Other studies have also indicated inhibition of IL-6-induced STAT3 activation by TNFα, IL-1β, IL-10, and LPS in other cell types (63, 142). As STAT3 is the main transcription factor in IL-6 signaling, inhibited STAT3

activation should result in decreased expression of IL-6 downstream target genes. However, in our previous study decreased STAT3 activation upon IL-6 treatment was accompanied by differentially modulated IL-6 downstream target genes; while SOCS3 was inhibited, MCP-1 expression was increased. As MCP-1 is a pro-inflammatory chemokine with atherogenic properties (156), the question was raised whether the inhibitory effect of IFNγ on IL-6-induced STAT3 activation could have divergent effects on the expression of anti and pro-inflammatory genes downstream of IL-6 signaling cascade in EC. The present study employed microarrays to study the transcriptional response to IL-6 without and with IFNγ pretreatment in EC. We hypothesized that part of the pro-inflammatory response of IL-6 would be enhanced by IFNγ and part of the anti-inflammatory response inhibited.

Materials and Methods

Reagents and cell culture

Human Micro-vascular EC were generously provided by Centers for Disease Control and Prevention (Atlanta, USA). Recombinant IFNγ and IL-6 were purchased from Sigma. One day before an experiment, the MCDB-131 (Invitrogen) medium supplemented with penicillin/streptomycin 1%, hEGF 0.1%, hydrocortisone 0.1%, and 10% fetal calf serum (Invitrogen) was replaced with MCDB-131 containing 0.5% FCS and penicillin/streptomycin 1%.

Experimental design

To identify the genes modulated by IL-6, IFNγ or IFNγ pretreatment + IL-6, four conditions have been chosen (n=4 for each condition) to study the gene profiling of HMEC cells in the presence or absence of IFNγ and IL-6 stimulation:

1. HMEC cells left untreated.
2. HMEC cells were treated with IFNγ (1ng/ml) for 3 hours.
3. HMEC cells were treated with IL-6 (50 U/ml) for 1 hour.
4. HEMC cells were pretreated with IFNγ (1ng/ml) for 2 hours and then with IL-6 (50 U/ml) for an additional hour.

RNA isolation and hybridization

Total RNA was isolated using TRIzol® (Gibco) according to the manufacturer's instruction and stored at -80 °C until use. Samples were sent to ServiceXS (Leiden, The Netherlands) for further processing. With the Agilent Bioanalyzer (lab on chip) the quality and integrity of the RNA samples was analyzed. The Illumina® TotalPrep™ RNA Amplification kit (Ambion, art. No. AM-IL1791) was used to synthesize biotine labeled cRNA. Concentration of the labeled cRNA was measured using the Nanodrop spectrophotometer. The amount of biotinylated cRNA that was hybridized onto the HumanRef-8 Expression BeadChip was 750 ng. In total 16 samples from 4 independent experiments (4 times 4) were used for RNA gene expression profiling. Two BeadChips were used, each one containing eight samples.

Data analysis

We developed software for the analysis of the raw bead data (T4Illumina). Briefly, the program first tested for each gene identifier whether there were beads that were outliers. Individual beads were excluded if the signal intensity was > median intensity + 2x median absolute deviation or < median intensity - 2x median absolute deviation. Next the mean intensity was calculated for each gene, and compared to the mean intensity of the negative controls. If the variance was not different between these means, a regular unpaired T-test was performed. If the variance was different, a T-test was performed for samples with unequal variance. The output of the program is a) a quality control file, b) a list with mean intensities per gene identifier, and c) a similar list, but with added p-values for the comparison to the negative controls. The mean intensities of all samples in all 4 groups were Log_2 SVT normalized using FlexArray (http://genomequebec.mcgill.ca/FlexArray/) and a CyberT test performed for each relevant comparison. The normalized data were then read by a second routine of T4Illumina. For each group, it was determined whether the average of each gene intensity of the group was different from the average negative controls in that same group ($P < 0.05$ was considered significant). A comparison using Cyber T-test between two samples was only performed on a specific gene, if one of the groups showed an average gene intensity larger than the negative controls ($P < 0.05$ was considered significant). Data of all groups was put together in one file (if one of the averages per gene was higher than the

negative control), and subjected to hierarchical cluster analysis using Expression Profiler (EBI). To identify the genes modulated by IL-6, IFNγ or IFNγ pretreatment + IL-6, intensity values of gene expression from 4 independent experiments were combined and ratios of IL-6 versus control, IFNγ versus control, and IFNγ-pretreatment+ IL-6 versus control were generated ($P < 0.05$ was considered significant).

Real time PCR

To confirm the microarray expression data, the expression of a few selected genes were tested by real time PCR (Applied Biosystems TaqMan® Assays-on-Demand™ Gene Expression Products) using pre-designed SOCS1, SOCS3, MCP-1, and 18S primers on the Applied Biosystems, 7900 HT Fast Real-Time PCR System. The amount of target gene in each subject was normalized to endogenous controls 18S (ΔCT). Gene expression is expressed as $(2^{-\Delta CT}*100) \pm$ S.D.

Results

IL-6-induced limited number of genes in EC

In contrast to IFNγ, IL-6 regulated relatively a small number of target genes in EC (821 vs. 170 respectively; fig. 1). However, the additive or synergistic interactions between IL-6 and IFNγ substantially increased the number of up as well as down-regulated genes (fig. 1). As indicated in the Venn diagram (fig. 1), there are 1485 genes regulated by IFNγ + IL-6. There is also an overlap between IL-6- and IFNγ- modulated genes. In fact, 30% of IL-6-regulated genes are also modulated by IFNγ (fig. 1).

IFNγ-pretreatment of HMEC cells differentially affected IL-6-regulated genes

Of the top 10, IFNγ-induced genes nine genes were known IFNγ-target genes, while eosinophil lysophospholipase-like (LOC400696) has not been reported previously (table 1). Among the most strongly induced genes by IL-6 (table 2), some genes can be found that are known IL-6 target genes including; SOCS3, pim1, Myc, and BCL3 (163). The expression of NLF2, was not reported previously. Interestingly, all genes that were reduced by IL-6, were

also reduced upon IFNγ stimulation (table 2). This was not true for IL-6-induced up-regulated genes. While MCP-1 and NLF-2 were increased by both IL-6 and IFNγ, Myc was decreased by IFNγ. IFNγ pretreatment of EC differentially affected the IL-6-regulated genes (table 3); the expression of some genes including SOCS3, MAP3K8, Myc and Id-1 was downregulated, while the expression of other genes including NLF2 and CCL2 was upregulated. Interestingly, there were also IFNγ target genes that were increased (e.g. CCL8, and CXCL11) in the presence of IL-6.

Quantitative PCR confirmed the microarray gene expression data

Using real time PCR we tested the expression of SOCS1, SOCS3, and MCP-1. Real time PCR confirmed the same pattern of SOCS1, SOCS3, and MCP-1 expression. However, in HMEC, MCP-1 is significantly increased by IFNγ, IL-6 or both in the microarray experiments, while it is only significantly increased in IFNγ-pretreatment + IL-6 condition according to the real time PCR data.

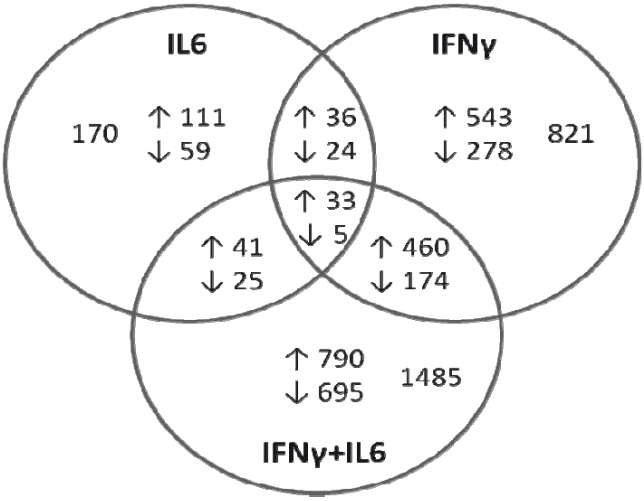


Fig. 1. Venn diagram illustrating the number of changes observed in the individual groups and overlap between the groups.

Table 1: The ten maximally up- and downregulated genes by IFNγ (data are expressed as fold change).

Transcript	Symbol	Definition	IL6	IFNγ	IL6+IFNγ
Reduced					
NM_005398	PPP1R3C	Homo sapiens protein phosphatase 1, regulatory (inhibitor) subunit 3C (PPP1R3C), mRNA.	0.78	**0.55**	0.50
NM_019058	DDIT4	Homo sapiens DNA-damage-inducible transcript 4 (DDIT4), mRNA.	0.99	**0.57**	0.52
NM_152330	FRMD6	Homo sapiens FERM domain containing 6 (FRMD6), mRNA.	0.89	**0.58**	0.56
NM_004431	EPHA2	Homo sapiens EPH receptor A2 (EPHA2), mRNA.	0.94	**0.61**	0.56
NM_145244	DDIT4L	Homo sapiens DNA-damage-inducible transcript 4-like (DDIT4L), mRNA.	0.88	**0.62**	0.60
NM_170600	SH2D3C	Homo sapiens SH2 domain containing 3C (SH2D3C), transcript variant 2, mRNA.	1.06	**0.62**	0.65
NM_001034	RRM2	Homo sapiens ribonucleotide reductase M2 polypeptide (RRM2), mRNA.	0.75	**0.63**	0.56
NM_153026	PRICKLE1	Homo sapiens prickle-like 1 (Drosophila) (PRICKLE1), mRNA.	0.85	**0.64**	0.63
NM_005841	SPRY1	Homo sapiens sprouty homolog 1, antagonist of FGF signaling (Drosophila) (SPRY1), transcript variant 1, mRNA.	1.68	**0.64**	0.88
NM_001945	HBEGF	Homo sapiens heparin-binding EGF-like growth factor (HBEGF), mRNA.	1.13	**0.64**	0.65
Induced					
NM_178232	HAPLN3	Homo sapiens hyaluronan and proteoglycan link protein 3 (HAPLN3), mRNA.	1.04	**6.32**	6.15
NM_207646	LOC400696	Homo sapiens eosinophil lysophospholipase-like (LOC400696), mRNA.	0.99	**6.82**	6.11
NM_139266	STAT1	Homo sapiens signal transducer and activator of transcription 1, 91kDa (STAT1), transcript variant beta, mRNA.	1.06	**6.96**	6.41
NM_213646	WARS	Homo sapiens tryptophanyl-tRNA synthetase (WARS), transcript variant 4, mRNA.	0.98	**8.28**	7.06
NM_005409	CXCL11	Homo sapiens chemokine (C-X-C motif) ligand 11 (CXCL11), mRNA.	1.09	**8.57**	9.32
NM_052941	GBP4	Homo sapiens guanylate binding protein 4 (GBP4), mRNA.	0.98	**9.65**	9.38
NM_002198	IRF1	Homo sapiens interferon regulatory factor 1 (IRF1), mRNA.	1.43	**9.99**	9.38
NM_002053	GBP1	Homo sapiens guanylate binding protein 1, interferon-inducible, 67kDa (GBP1), mRNA.	0.98	**10.70**	10.34
NM_002163	IRF8	Homo sapiens interferon regulatory factor 8 (IRF8), mRNA.	1.04	**12.04**	11.16
NM_001565	CXCL10	Homo sapiens chemokine (C-X-C motif) ligand 10 (CXCL10), mRNA.	0.99	**12.91**	13.00

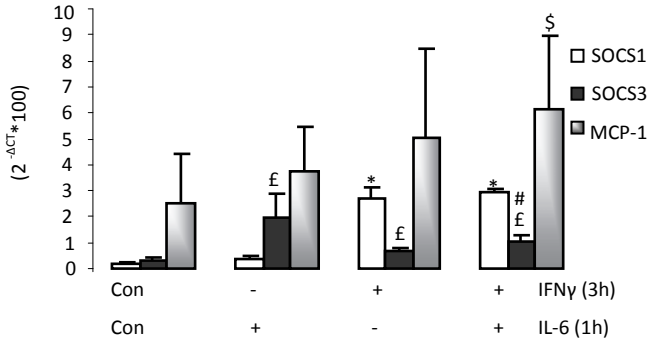
Table 2: The ten maximally up- and downregulated genes by IL6 (data are expressed as fold change).

Transcript	Symbol	Definition	IL6	IFNγ	IL6+IFNγ
Reduced					
NM_005398	PPP1R3C	Homo sapiens protein phosphatase 1, regulatory (inhibitor) subunit 3C (PPP1R3C), mRNA.	**0.78**	0.55	0.50
NM_006298	ZNF192	Homo sapiens zinc finger protein 192 (ZNF192), mRNA.	**0.84**	0.82	0.82
NM_001008565	NUPL1	Homo sapiens nucleoporin like 1 (NUPL1), transcript variant 3, mRNA.	**0.86**	0.81	0.88
NM_013308	GPR171	Homo sapiens G protein-coupled receptor 171 (GPR171), mRNA.	**0.87**	0.91	0.93
NM_002655	PLAG1	Homo sapiens pleiomorphic adenoma gene 1 (PLAG1), mRNA.	**0.87**	0.85	0.83
NM_145110	MAP2K3	Homo sapiens mitogen-activated protein kinase kinase 3 (MAP2K3), transcript variant C, mRNA.	**0.88**	0.84	0.81
NM_001025107	ADAR	Homo sapiens adenosine deaminase, RNA-specific (ADAR), transcript variant 4, mRNA.	**0.88**	0.86	0.88
NM_178009	DGKH	Homo sapiens diacylglycerol kinase, eta (DGKH), transcript variant 2, mRNA.	**0.88**	0.92	0.91
NM_022894	PAPOLG	Homo sapiens poly(A) polymerase gamma (PAPOLG), mRNA.	**0.88**	0.90	0.91
NM_020666	CLK4	Homo sapiens CDC-like kinase 4 (CLK4), mRNA.	**0.89**	0.86	0.86
Induced					
NM_005204	MAP3K8	Homo sapiens mitogen-activated protein kinase kinase kinase 8 (MAP3K8), mRNA.	**1.75**	1.09	1.38
NM_002648	PIM1	Homo sapiens pim-1 oncogene (PIM1), mRNA.	**1.84**	1.16	1.48
NM_002467	MYC	Homo sapiens v-myc myelocytomatosis viral oncogene homolog (avian) (MYC), mRNA.	**1.89**	0.74	1.06
NM_005178	BCL3	Homo sapiens B-cell CLL/lymphoma 3 (BCL3), mRNA.	**1.96**	1.65	2.17
NM_003407	ZFP36	Homo sapiens zinc finger protein 36, C3H type, homolog (mouse) (ZFP36), mRNA.	**2.07**	1.66	2.51
NM_001007595	NLF2	Homo sapiens nuclear localized factor 2 (NLF2), mRNA.	**2.27**	2.46	3.10
NM_002982	MCP-1	Homo sapiens monocyte chemoattractive protein 1, mRNA.	**2.35**	2.60	3.23
NM_181353	ID1	Homo sapiens inhibitor of DNA binding 1, dominant negative helix-loop-helix protein (ID1), transcript variant 2, mRNA.	**2.48**	0.68	1.02
NM_005195	CEBPD	Homo sapiens CCAAT/enhancer binding protein (C/EBP), delta (CEBPD), mRNA.	**2.97**	2.01	2.81
NM_003955	SOCS3	Homo sapiens suppressor of cytokine signaling 3 (SOCS3), mRNA.	**3.92**	1.53	2.27

Table 3. IL-6 target genes reduced or increased by IFNγ-pretreatment of HMEC cells(data are expressed as fold change).

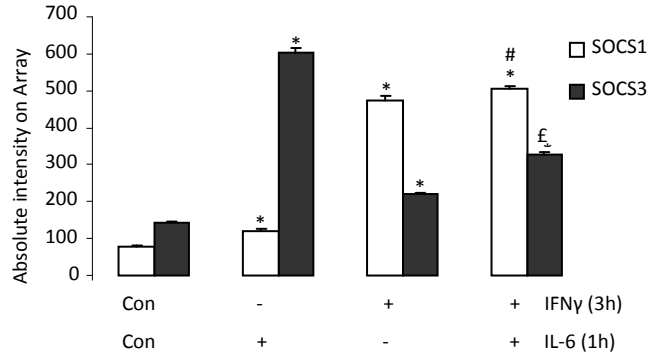
Transcript	Symbol	Definition	IL6	IFNγ	IL6+IFNγ
Reduced					
NM_052815.1	IER3	Homo sapiens immediate early response 3 (IER3), transcript variant long, mRNA.	**1.4**	0.7	**0.8**
NM_033027.2	AXUD1	Homo sapiens AXIN1 up-regulated 1 (AXUD1), mRNA.	**1.4**	0.8	**0.8**
NM_139314.1	ANGPTL4	Homo sapiens angiopoietin-like 4 (ANGPTL4), transcript variant 1, mRNA	**1.6**	0.9	**0.9**
NM_005985.2	SNAI1	Homo sapiens snail homolog 1 (drosophila), mRNA	**1.6**	0.9	**1.2**
NM_005841	SPRY1	Homo sapiens sprouty homolog 1, antagonist of FGF signaling (Drosophila) (SPRY1), transcript variant 1, mRNA.	**1.7**	0.6	**0.9**
NM_003670.1	BHLHB2	Homo sapiens basic helix-loop-helix domain containing, class B, 2 (BHLHB2), mRNA.	**1.7**	0.7	**1.0**
NM_005204	MAP3K8	Homo sapiens mitogen-activated protein kinase kinase kinase 8 (MAP3K8), mRNA.	**1.8**	1.1	**1.4**
NM_002467	MYC	Homo sapiens v-myc myelocytomatosis viral oncogene homolog (avian) (MYC), mRNA.	**1.9**	**0.7**	**1.1**
NM_181353	ID1	Homo sapiens inhibitor of DNA binding 1, dominant negative helix-loop-helix protein (ID1), transcript variant 2, mRNA.	**2.5**	0.7	**1.0**
NM_003955	SOCS3	Homo sapiens suppressor of cytokine signaling 3 (SOCS3), mRNA.	**3.9**	1.5	**2.3**
Increased					
NM_005623	CCL8	Homo sapiens chemokine (C-C motif) ligand 8 (CCL8), mRNA.	**1.1**	5.9	**7.8**
NM_0054409	CXCL11	Homo sapiens chemokine (C-X-C motif) ligand 11 (CXCL11), mRNA.	**1.1**	8.6	**9.3**
NM_006084.3	ISGF3G	Homo sapiens interferon-stimulated transcription factor 3, gamma 48kDa (ISGF3G), mRNA.	**1.1**	2.8	**3.1**
NM_001030287.1	ATF3	Homo sapiens activating transcription factor 3 (ATF3), transcript variant 3, mRNA.	**1.3**	3.0	**3.3**
NM_003155.2	STC1	Homo sapiens stanniocalcin 1 (STC1), mRNA.	**1.4**	1.5	**1.9**
NM_003745	SOCS1	Homo sapiens suppressor of cytokine signaling 1 (SOCS1), mRNA.	**1.5**	6.0	**6.5**
NM_002089	CXCL2	Homo sapiens chemokine (C-X-C motif) ligand 2 (CXCL2), mRNA.	**1.7**	1.6	**2.2**
NM_003407	ZFP36	Homo sapiens zinc finger protein 36, C3H type, homolog (mouse) (ZFP36), mRNA.	**2.1**	**1.7**	**2.5**
NM_001007595	NLF2	Homo sapiens nuclear localized factor 2 (NLF2), mRNA.	**2.3**	2.5	**3.1**
NM_002982	MCP-1	Homo sapiens monocyte chemoattractive protein 1, mRNA.	**2.3**	2.6	**3.2**

A



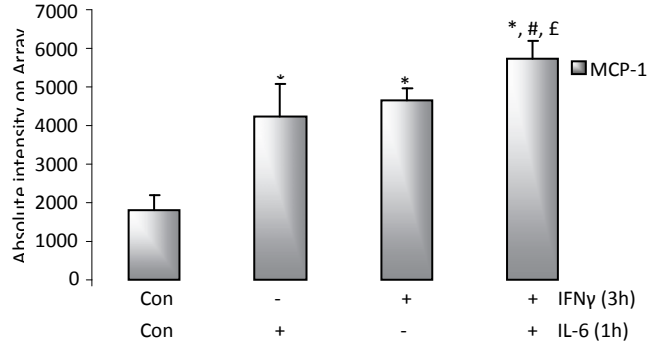
** vs. Con. ; P < 0.05, data compared with Anova On Ranks / Dunn's , £ vs. Con; P< 0.001, # vs. IL-6 (50U/ml); P< 0.01, data compared with One Way Anova / Student Newman Keuls method, $ vs. Con; P< 0.05, data compared with Anova On Ranks / Dunn's method*

B



** vs. Con., # vs. IFNγ (1 ng/ml); ; £ vs. IL-6 (50 U/ml) ; P< 0.001, data compared with One Way Anova / Student Newman Keuls method*

C



** vs. Con.; P< 0.001, # vs. IFNγ (1 ng/ml); ; £ vs. IL-6 (50 U/ml) ; P< 005, data compared with One Way Anova / Student Newman Keuls method*

Fig. 2. Real time PCR and microarray gene expression yielded similar results. Quantitative real time PCR (A) and microarray (B-C) expression data for the genes coding for SOCS and MCP-1. Real time PCR data (A) are from 6 independent experiments and are presented as mean ($2^{-\Delta CT}$*100) ± S.D. Microarray data (B-C) are mean intensity values (normalized as described under material and methods) of 4 independent experiments ± S.D.

Discussion

Previously we have reported that IFNγ-pretreatment of HMEC cells led to inhibition of IL-6-induced STAT3 activation, which was accompanied by decreased SOCS3 and increased MCP-1 expression (chapter 4). These data suggested that the inhibitory effect of IFNγ on IL-6 signaling might differentially modulate IL-6 target genes expression. Since MCP-1 has proatherogenic effects we suggested that part of the pro-inflammatory response of IL-6 would be enhanced by IFNγ and part of the anti-inflammatory response inhibited. Using mRNA gene expression profiling we characterized the transcriptome of IFNγ and IL-6 as well as the effect of IFNγ on IL-6 transcriptome in HMEC cells.

IL-6 modulated a relatively small number of genes in EC (fig. 1). Similarly, IL-10 induces a limited number of genes in monocytes, which are not believed to be responsible for the anti-inflammatory effects of IL-10 (163). Instead, it has been suggested that anti-inflammatory effects of IL-10 is mostly related to suppression of inflammatory genes induced by pro-inflammatory stimuli rather than induction of new genes (163). The relevance of this hypothesis for anti-inflammatory effects of IL-6 needs to be clarified. However, our data indicated that IL-6 indeed decreased the expression of the most IFNγ-target genes listed in table 1. In contrast to IL-10, biological responses to IFNγ are mainly modulated by the regulation of gene expression, mediated by Jak/STAT pathway (157). In accord, IFNγ stimulation of HMEC cells led to modulation of a great number of genes (fig. 1). Interestingly, IFNγ treatment of EC prior to IL-6, resulted in even more significantly regulated genes, indicative a synergistic or additive effect of IFNγ and IL-6 (fig. 1). To confirm the microarray gene expression data, the expression of SOCS1, SOCS3 and MCP-1 were tested with real time PCR, which indicated similar results. However, MCP-1 was not significantly induced by IL-6 or IFNγ in real time PCR experiments. This might be related to higher variance in MCP-1 expression in those experiments.

Most of the top-10 IFNγ-induced genes listed in table 1 are known to inhibit proliferation and to induce apoptosis (e.g. GBP-1 (164), IRF-1 (165)) or increase the adhesiveness of EC (e.g. CXCL11 (166), IRF-1 (167) IP-10(168)). Among the maximally induced genes upon IL-6 stimulation was SOCS3, which is a known IL-6 target gene and has specific role in inhibition of IL-6 signaling (63). Furthermore, in the absence of SOCS3, IL-6 has anti-

inflammatory effects in macrophages, which is related to prolonged activation of STAT3 (63, 153). Some IL-6-induced genes (table 2) have potentially pro-inflammatory and pro-atherogenic properties. For instance, MAP3K8, which is required for CD40-induced NF-Kβ activation (169). NLF2, is a newly identified inflammatory gene induced by IL-1β in EC (170). Here we report the induction of NLF2 by both IL-6 and IFNγ in EC. NLF2 is believed to increase vascular permeability in acute inflammation (170). MCP-1 is a chemokine with atherogenic properties. MCP-1 enhances atherosclerotic plaques formation by mediating monocyte/macrophage infiltration into arterial wall and foam cell formation (156). CEBPD or NF-IL-6-beta is also involved in NF-Kβ signaling (171) and induced also by other inflammatory cytokines such as IL-1β (172) and TNFα (173). IL-6 induces CEBPD in a STAT3 dependent manner in hepatocytes (174). CEBPD expression is associated with acute phase gene induction and plays an important role in LPS induced inflammation of mouse macrophages (175).

Other IL-6 induced genes (table 2) have more protective and proliferative effects. Zinc finger protein 36 (ZFP36) is induced in EC in response to inflammatory cytokines such as IL-1β (176) and has been shown to bind to and accelerate mRNA degradation, as already demonstrated for TNF-α and granulocyte-macrophage colony-stimulating factor (GM-CSF) mRNAs (177). proto-oncogene BCL3 could have anti-inflammatory effects by inhibiting the NF-Kβ (178). Id-1 is an interesting target of IL-6 in the scope of this study. Id-1 promotes EC proliferation, angiogenesis and EC migration and delay EC senescence (179, 180). Pim1 and Myc are STAT3-dependent IL-6 targets that promote proliferation and have anti-apoptotic effects (181).

Pretreatment of EC with IFNγ, considerably affected IL-6-induced genes. A fast overview of the genes listed in table 1-3 suggests at least 3 sets of genes:

The first group includes IL-6 target genes that are inhibited in the presence of IFNγ such as: SOCS3, Id-1, Pim-1, MAP3K8, BHLHB2, and Myc (table 3). As the expression of these genes were preceded by inhibited IL-6-induced STAT3 activation. It could be suggested that they are STAT3-target genes. Indeed previous studies indicated the STAT3-dependency of Myc (182), SOCS3 (183), Pim1 and Myc (181). This indicates that the expressions of IL-6 target genes that are STAT3-dependent are diminished in the presence of IFNγ in a SOCS3-dependent manner. Importantly, some of the STAT3-target genes including Id1, Pim-1, SOCS3

and Myc are also induced immediately by IFNγ in STAT1$^{-/-}$ cells (157, 184). It has been suggested that immediate- early genes, induced by IFNγ in STAT1$^{-/-}$ cells, play important roles in cell proliferation (185). This suggests that induction of some immediate IFNγ target genes could be STAT3-dependent. As the role of STAT3 as a part of IFNγ signaling has not yet been defined, we suggest that the induction of those genes could be mediated by fast activation of STAT3 upon IFNγ stimulation (chapter 4).

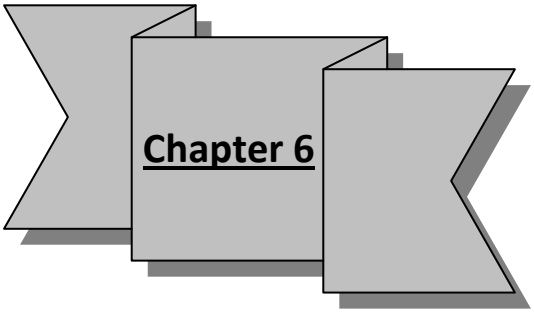
The second group of genes shows an expression patterns like MCP-1. There are genes that are induced by both IL-6 and IFNγ, and their expressions are increased upon IFNγ + IL-6 stimulation that includes MCP-1, NLF-2, ZFP36, and CXCL2. As the expression of these genes does not follow the fashion of IL-6-induced STAT3 activation in the presence of IFNγ, it could be suggested that these genes are STAT3-independent. A comparative study in wild type and STAT1$^{-/-}$ cells indicated that MCP-1 can be induced in both cell types upon IFNγ stimulation (157), indicating MCP-1 also as a STAT1-independent gene. Although, the STAT-dependency or independency of the other genes in this class needs to be identified, we suggest that the expression of these genes might not be STAT1 or 3 dependent. This idea is also supported by the fact that the expression of these genes upon IFNγ stimulation is much lower than STAT1-dependent target genes of IFNγ such as IP-10 and IRF-1 (157). Furthermore, NLF-2 is reported to be induced by IL-1β, and TNFα in EC (172, 173), and the role of STAT as a part of IL-1β and TNFα is not well understood.

The third group of genes is IFNγ target genes that are increased in the presence of IL-6 that includes CXCL11, CCL8, ISGF3G, ATF3 and SOCS1. The expression of CCL8, CXCL11, and SOCS1 have been demonstrated to be STAT1-dependent upon IFNγ stimulation (157, 186), suggesting that IL-6 might in someway potentiate the transcriptional activity of STAT1. Although, we could not demonstrate further activation of IFNγ-induced STAT1 activation upon IL-6 stimulation (chapter 4). This does not rule out the possibility that IL-6 could potentiate the transcriptional activity of STAT1. This idea is supported by the fact that STAT1 activation in EC was not concentration-dependent upon 1-5 ng/ml IFNγ, suggesting that STAT1 is maximally activated even with 1ng/ml IFNγ. However, it is not possible to classify these genes as only STAT1 dependent genes, especially because IL-6 inhibited some known STAT1-dependent IFNγ target genes such as IRF1 (table 1).

In sum, gene expression profiling of IL-6 in EC indicated that IL-6 induces a limited number of inflammatory as well as protective genes in EC. IL-6 stimulation of EC in the presence of IFNγ, inhibited the expression of some IL-6 target genes with protective and proliferative effects such as Id-1, Pim-1, and Myc, while the expression of some other genes in particular chemokines were increased, which might potentiate migration of leukocyte through EC layer and promote atherosclerosis. Our data suggest that IL-6 and IFNγ induce genes in STAT-dependent and independent fashions. Unfortunately, our present knowledge is limited to the information regarding the IFNγ-induced transcriptome in wild type and $STAT1^{-/-}$ cells. This study highlights the importance of further studies to define the role of other transcription factors downstream of IL-6 and IFNγ signaling.

Acknowledgements

This work was supported by grants from Dutch kidney foundation (NSN C03-2062), a grant by the Genzyme Renal Innovations Program (GRIP) and a grant by the Dutch Heart Foundation (2005B192).



IL-6 induced acute phase response in the liver is also STAT1 dependent

Mehdi Rastmanesh[1], Branko Braam[1,2, 3], Marie-Jose Goumans[4], Yaming Wang[5] , David E. Levy[5] , and Hans A.R. Bluyssen[6]

[1] Department of Nephrology and Hypertension, University Medical Center Utrecht, the Netherlands. [2] Division of Nephrology and Immunology, Dept. Medicine, Univ. of Alberta, Edmonton, Canada and [3] Dept. Physiology, Univ. of Alberta, Edmonton, Canada. [4] Department of experimental Cardiology, University Medical Center Utrecht, The Netehrlands, [5] Department of Pathology, New York University , New York, USA, [6] Laboratory of Human Molecular Genetics, Institute of Molecular Biology and Biotechnology, Faculty of Biology, Adam Mickiewicz University, Poznan, Poland.

A pilot study

Abstract

STAT3 carries the most characterized functions of IL-6 including induction of acute phase response (AP) in the liver. However, IL-6 stimulation of hepatocytes leads to activation of both STAT1 and STAT3. The role of STAT1 as a part of IL-6 signaling and in particular induction of acute phase response is not well studied. In the present pilot study we measured the expression of two well known AP genes; serum amyloid-A1 (SAA1) and haptoglobin (Hp) in wild type and $STAT1^{-/-}$ mice to test the hypothesis that STAT1 plays an active role in induction of AP genes in the liver upon IL-6 stimulation.

Wild type and $STAT1^{-/-}$ balb/c mice were treated with IL-6 together with IL-6 soluble receptor. STAT activation in the liver was studied by western analysis, gene expression was assessed by real time PCR.

IL-6 induced STAT3 activation in the liver that was followed by SOCS1, SOCS3, SAA1 and Hp expression in the liver. IL-6-induced STAT3 activation and gene expression was time-dependent, SOCS1 was maximally induced after 1 hour, SOCS3 and Hp after 2 hours and SAA1 after 4 hours. SAA1 expression was substantially decreased in $STAT1^{-/-}$ mice.

IL-6 stimulation of wild type and $STAT1^{-/-}$ mice for four hours significantly increased SAA1 expression in wild type but not in $STAT1^{-/-}$ mice. Decreased SAA1 expression in $STAT1^{-/-}$ mice indicates the importance of STAT1 in IL-6-induced SAA1 expression in the liver. STAT1 activation and the role of STAT1 in SOCS and Hp and other IL-6 target genes expression in the liver needs to be studied at other time-points.

Introduction

IL-6 has both pro- (95) and anti-inflammatory (187) effects as well as pro- (95) and anti-atherogenic (139) effects. IL-6 promotes inflammatory responses through different mechanisms including induction of an acute phase (AP) response in the liver (95). IL-6-induced AP proteins, e.g. C-reactive protein (CRP), and serum amyloid A (SAA) are strong and consistent inflammatory markers that are associated with cardiovascular events (188, 189). In mice, SAA represent the main AP protein, while CRP is the minor in response to IL-6 (188, 190). In addition to its role as a marker of inflammation, SAA can be found in atherosclerosis lesions at all stages and can also function as a chemo-attractant for macrophages in mice (191). However, there are also other IL-6-induced AP proteins that have protective effects. For instance haptoglobin (Hp) that gives protection against reactive oxygen species (192).

At signaling level, IL-6 binds to the IL-6 receptor-α (IL-6Rα; gp80) that is not involved in signal transduction events. Signaling is triggered by the IL-6/IL-6Rα-induced homodimerization of signal transducer gp130 subunits (gp130) (193), followed by Janus Kinase (Jak) activation, receptor phosphorylation and, finally, activation of signal transducer and activator of transcription 1 (STAT1) and STAT3 (75, 194). While the role of STAT1 as a part of IL-6 signaling is not well understood, STAT3 carries the most well characterized effects of IL-6 including induction of acute phase (AP) response in the liver (75, 195) and inhibition of cytokine production by macrophages (140, 141). However, induction of some AP genes (e.g. complement component-3 (C3)) are not impaired in conditional $STAT3^{-/-}$ mice ($STAT3^{-/-}$ in the liver and macrophages) upon IL-6 treatment (75). Furthermore, we have previously demonstrated that pretreatment of endothelial cells with IFNγ diminishes IL-6-induced STAT3 activation (chapter 4). Using mRNA micro-array gene profiling, we have reported that inhibition of IL-6-induced STAT3 activation is accompanied by inhibition of some IL-6 target genes, while the expression of other genes are unaffected or even increased (in particular some IFNγ target genes)(chapter 5). Together, these observations suggest that other transcription factor(s) should also be involved in IL-6 signaling.

It is now clear that in the absence of STAT3, IL-6 is able to signal through STAT1 (152), indicating STAT1 as an important downstream component of IL-6 signaling. In fact, there are two tyrosine modules within the cytoplasmic tail of gp130 that mediate STAT1

activation, which is independent from STAT3 recruitment and activation (194). These observations suggest that STAT1 might play an important role in IL-6 signaling, and could carry some IL-6 effects. The present study was, therefore, initiated to test the hypothesis that STAT1 plays an active role in IL-6-induced AP response in the liver. To test this hypothesis we used wild type and $STAT1^{-/-}$ mice to measure the expression of SAA1 (a member of SAA family (196)), which is reported to be totally defective in $STAT3^{-/-}$ hepatocytes and Hp, that is not substantially affected in $STAT3^{-/-}$ hepatocytes upon IL-6 stimulation (75).

Material and Methods

Animal models

Wild type, $STAT1^{-/-}$ Balb/c mice, recombinant human IL-6, and recombinant human IL-6 soluble receptor (IL-6 SR) were generously provided by Dr. D.E. Levy (School of Medicine, NYU, USA). The establishment of $STAT1^{-/-}$ mice has been described previously (197). Animals received IL-6 and IL-6 SR or PBS intra-peritoneally. After desired treatment duration animals were sacrificed and their liver were isolated, rinsed briefly with PBS and frozen on dry ice and stored at -80°C. All animal experiments were done at the Department of Pathology, Dr. D.E. Levy's Lab and in accordance with the animal guidelines of the New York University.

Western analysis

Frozen livers were lyzed by homogenization in a buffer containing complete EDTA-free protease inhibitor cocktail (Roche), NaF (40 mM), $NaVo_4$ (4 mM), and Triton-X100 (1%). The protein concentration was determined by detergent compatible (DC) protein assay (Bio-Rad). Proteins were fractionated by sodium dodecyl sulfate-polyacrylamide gel (7.5%) electrophoresis and transferred to nitrocellulose membrane (Roche). After blocking in ECL-blocking buffer the membrane was either incubated with polyclonal antibodies for P-Tyr STAT1 and 3 (1:1000) or specific monoclonal antibodies for STAT1, STAT3 (1:5000). After 3 times washing with PBS-Tween (PBST) the membrane was incubated with goat anti-mouse, goat anti-rabbit or rabbit anti-goat HRP conjugated (1: 1000) antibody. Next, the membrane was washed 2 times with PBST and once with PBS and bound proteins were detected with enhanced chemoluminescence (ECL, Amersham).

Gene expression assay

Frozen tissues were lyzed by homogenization in TRIzol® (Gibco). Total RNA was isolated using TRIzol®, one microgram of total RNA was used for cDNA synthesis by using oligo(dT) primer. Relative abundances of specific mRNA sequences were determined by real-time fluorescent PCR by using Syber green (Molecular Probes) by comparison with a standard curve generated by serial dilution of a cDNA sample containing abundant target sequences and normalized to the expression of GAPDH, and presented as the mean ($2^{-\Delta CT}$*100) ± SD. All PCR reactions were performed in duplicate. Sequences of primers used are available on request.

Statistics

Data (in fig. 2) were compared using one Way ANOVA and the Studentized Newman Keuls post-hoc test, which was performed with SigmaStat (version 3.5, Systat Software Inc, San Jose, Ca). $P < 0.05$ was considered to be significant.

Results

IL-6 activates STAT3 and induces AP genes in a time-dependent manner in mice liver

Wild type female balb/c mice (n=1 in each time-point) were treated with IL-6 (IP,1µg/g body weight) plus IL-6sR (5ng/g body weight) for up to 6 hours. IL-6 induced only STAT3 activation in the liver, which was detectable at the first time-point (1 hour) and decreased after 2 hours (fig. 1A; n=1). This was accompanied by SOCS1 and SOCS3 induction. However, while SOCS1 expression indicated a peak at 1 hour, SOCS3 was maximally increased after 2 hours (fig. 1B; n=1). Furthermore, IL-6 induced serum amyloid-A1 (SAA1) and haptoglobin (Hp) expression. The expression of SAA1 and Hp was also time-dependent. Hap was maximally induced after two hours, while SAA1 peak was measured at 4 hours (fig. 1C; n=1).

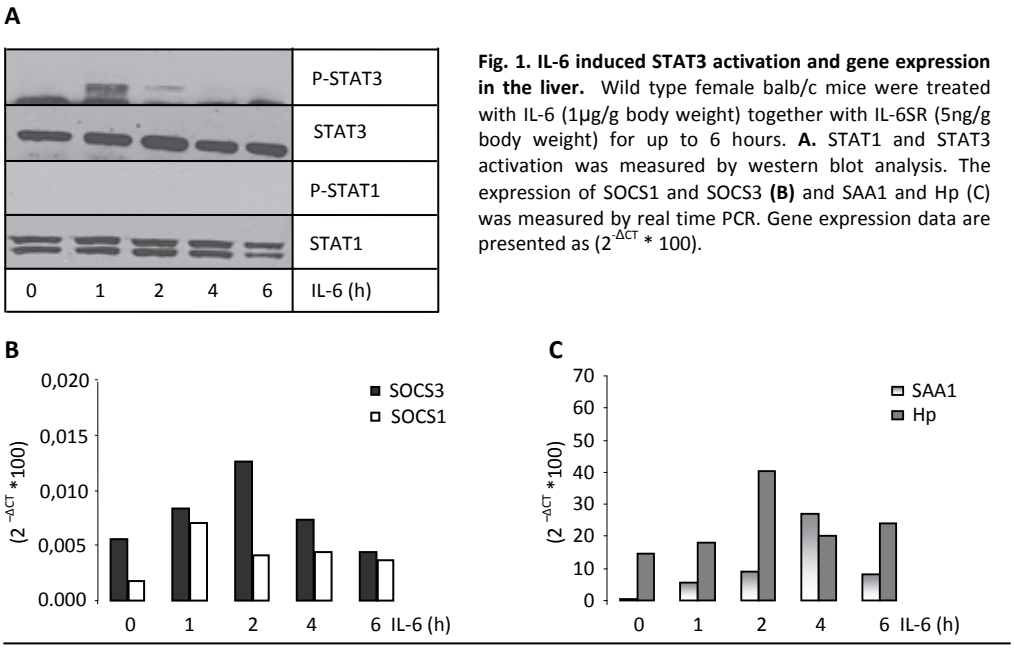
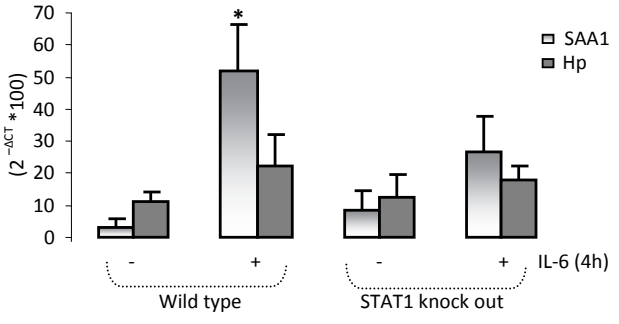


Fig. 1. IL-6 induced STAT3 activation and gene expression in the liver. Wild type female balb/c mice were treated with IL-6 (1µg/g body weight) together with IL-6SR (5ng/g body weight) for up to 6 hours. **A.** STAT1 and STAT3 activation was measured by western blot analysis. The expression of SOCS1 and SOCS3 **(B)** and SAA1 and Hp (C) was measured by real time PCR. Gene expression data are presented as ($2^{-\Delta CT} * 100$).

STAT1 plays an important role in IL-6-induced SAA expression in the liver

Next wild type and STAT1$^{-/-}$ balb/c mice were treated with IL-6 (IP,1 µg/g body weight) plus IL-6sR (5ng/g body weight) or PBS for 4 hours (n=3 in each condition). At this time point we could not detect STAT activation (data not shown), as it could be expected

according to the time-course study (fig. 1A). However, IL-6 induced SAA1 and Hap in the liver. There was no significant difference in Hap expression between wild type and STAT1$^{-/-}$ mice in the presence or absence of IL-6 at this time point. In contrast, SAA1 was only significantly increased in wild type balb/c mice and its expression was substantially decreased in STAT1$^{-/-}$ mice, indicating the importance of STAT1 in SAA1 induction upon IL-6 stimulation of the liver (fig. 2; n=3).



* vs. wild type con. P < 0.01; data were analyzed by One Way Anova / Student-Newman-Keuls test

Fig. 2. STAT1 is necessary for maximal induction of SAA1 in the liver upon IL-6 stimulation. Wild type and STAT1-/- balb/C mice were treated with IL-6 (1µg/g body weight) together with IL-6SR (5ng/g body weight) for 4 hours. Each condition contained 3 mice (2 female and 1 male) and in total 12 mice were used in this experiment. The expression of SAA1 and Hp was measured by real time PCR. Data are presented as ($2^{-\Delta CT}$ * 100) ± S. D.

Discussion

The present pilot study was initiated to study the role of STAT1 in IL-6 signaling and in particular IL-6-induced AP response in the liver. We used STAT1$^{-/-}$ mice model, which is a well-characterized model (197) and give the opportunity to study the STAT1 function in a knocked out system, which is much more convenient than knocked down models (e.g. by RNA interference), particularly, because STAT1 is abundantly present in different cell types (198) (see also chapter 4). Besides in contrast to conditional STAT3$^{-/-}$ mice, in which STAT3 is removed from hepatocytes and macrophages (75), in STAT1$^{-/-}$ mice, STAT1 is knocked out in all tissues. This makes STAT1$^{-/-}$ mice a very interesting model to study the role of STAT1 in IL-6 signaling in other tissues as well. In particular STAT1$^{-/-}$ mice is an outstanding model for studying the role of STAT1 in IL-6 signaling (in the presence of absence of IFNγ) in endothelial cells, which has not yet studied.

The IL-6 system promotes inflammatory events partially through induction of an acute phase response in the liver (95). In this regard, STAT3 suggested playing the central role

(75). Indeed, STAT3 was first described as a transcription factor in IL-6–stimulated hepatocytes, capable of selectively interacting with an enhancer element in the promoter of acute-phase genes (182).

However, the induction of some AP genes (e.g. C3) is not affected or just affected at later time points (e.g. Hp) in conditional $STAT3^{-/-}$ mice (75), indicating that other transcription factor(s) should be involved to cover all transcriptional activities of IL-6. Importantly, STAT1 activation is increased in $STAT3^{-/-}$ cells (75, 152), introducing STAT1 as an important transcription factor in IL-6 signaling. In fact, two of the four tyrosine modules within the cytoplasmic tail of gp130 are able to mediate STAT1 activation, which is independent of STAT3 (which means that STAT3 is not necessary for STAT1 binding to gp130) (194). STAT1 and STAT3 may also compete with each other to bind to the cytoplasmic domain of the receptor, for example in COS7 cells that has low endogenous amount of STAT3, IL-6 stimulation preferentially activates STAT1 (194). Together, these findings indicate STAT1 as a potential transcription factor candidate in IL-6 signaling.

In line with previous studies (75, 194), we could indicate STAT3 activation upon IL-6 stimulation in the liver, which was accompanied by increased SOCS1, SOCS3, SAA1 and Hap expression in a time-dependent fashion. Although, we could not detect STAT1 activation in wild type mice (which could be related to the time-points used in this study), the expression of SAA1 was substantially decreased in $STAT1^{-/-}$ mice, indicating the importance of STAT1 in SAA1 induction in the liver upon IL-6 stimulation. A previous report indicated that SAA1 expression is totally defective in conditional $STAT3^{-/-}$ mice (75). Our data indicated that STAT1 is also necessary for maximal induction of SAA1. In fact, IL-6 stimulation of hepatocytes leads to activation of both STAT1 and STAT3 (194). Activated STAT1 and STAT3 could make homo or heterdimers, both capable of translocating into the nucleus and initiating of transcription (199). It is therefore, possible to imagine that STAT3/STAT3, STAT1/STAT1 and STAT1/STAT3 dimers are capable of binding to SAA1 promoter.

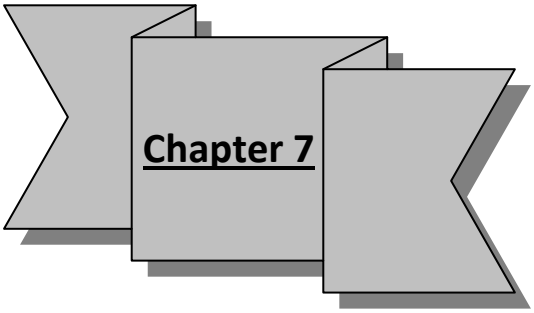
As mentioned before IL-6 has both pro and anti-inflammatory effects. STAT3 is known as an anti-inflammatory transcription factor, important in IL-10 signaling (200). Other studies even suggest that STAT3 activation per se has anti-inflammatory effects (201). In accord, Conditional $STAT3^{-/-}$ animals (in macrophages and liver) show hyperproduction of cytokines and are susceptible to endotoxic shock (8). In contrast to STAT3, STAT1 is the major

transcription factor activated by IFNγ signaling and carries the pro-inflammatory effects of IFNγ and has opposing effects comparing to STAT3 (150-152). Taken together, it is interesting to suggest that STAT3 predominantly carries the anti-inflammatory, and STAT1 might carry some pro-inflammatory effects of IL-6 (in distinct cell types and under certain circumstances).

This study provides preliminary data regarding the role of STAT1 in IL-6 signaling, confirmed by defective SAA1 expression in STAT1$^{-/-}$ mice upon IL-6 treatment. Unfortunately, we were not able to test the expression of SOCS and Hp at their suitable time points in wild type and STAT1$^{-/-}$ mice (fig. 1B-C). Also we could not detect STAT1 activation, which needs to be studied in other time points and with different concentrations of IL-6. Furthermore, we have previously indicated, diminished IL-6-induced STAT3 activation in endothelial cells in the presence of IFNγ. This was accompanied by decreased expression of some STAT3-dependent protective genes and simultaneously by increased some IFNγ-induced chemokines such as CCL8 and CXCL11, which play important role in atherosclerosis development by attracting the mononuclear cells through the endothelial cell layer (202). As induction of CCL8 and CXCL11 is STAT1-dependent (186), we suggested that IL-6 could in someway increase transcriptional activity of STAT1. In this regard, the STAT1$^{-/-}$ mice will be the suitable model for studying the role of STAT1 in IL-6 signaling in endothelial cells in the presence or absence of IFNγ, and could clarify whether STAT1 carries some pro-inflammatory and pro-atherogenic effects of IL-6.

Acknowledgments

We gratefully thank dr. Levy for his support and collaboration. This work was supported by a grant from Dutch Kidney Foundation.



Summary, discussion and perspectives

Summary

Atherosclerosis is the major cause of morbidity and mortality in renal patients (1) and a major health concern in western countries per se. Recent studies point to the important role of inflammation as an underlying cause of atherosclerosis (1). Importantly drugs that limit the inflammation lower the incidence of atherosclerosis as well (12). This indicates the importance of molecules that are able to control inflammation. Inflammation in renal patients is characterized by increased plasma levels of some inflammatory cytokines in particular TNFα, IL-10, IL-1β, and IL-6 (1, 7).

Cytokines may have both pro-inflammatory and anti-inflammatory properties. A dysbalance between these two types of cytokines is involved in progression of several inflammatory diseases and possibly in enhanced/accelerated atherosclerosis in renal patients (7, 33). If the anti-inflammatory cytokines are not able to counteract the effects of pro-inflammatory cytokines, other molecules that might diminish or inhibit the pro-inflammatory process will be of great importance. Suppressors of cytokine Signaling (SOCS; a family of 8 proteins: SOCS1-7 and CIS-1) are inducible inhibitors of cytokine signaling (40). They are induced by cytokines and in turn in a negative feedback loop they inhibit signaling initiated by the cytokines. This suggests that SOCS proteins not only inhibit the inflammatory signals initiated by the cytokines, but also indicate that systemic inflammation affects the intracellular signaling. Furthermore, the function of SOCS protein will not necessarily lead to diminished inflammation. SOCS proteins could also inhibit the anti-inflammatory and protective signals initiated by anti-inflammatory stimuli as well (72, 73), and in this way they may participate in progression of inflammation and inflammatory diseases. In fact, renal failure is accompanied by resistance to some beneficial stimuli such as EPO and GH (69-71). In this regard, in vitro and animal studies suggested that SOCS could be involved in GH and EPO-resistance (72, 73).

Data presented in this thesis can be divided into *in vivo* and *in vitro* studies. At first we tested the hypothesis that increased inflammation in patients with renal failure should be related to increased SOCS expression in peripheral blood mononuclear cells that are in direct contact with systemic inflammation, are involved in production of inflammatory cytokines, and are involved in atherosclerosis development **(chapter 2-3)**. Indeed, in chronic kidney

disease patients (CKD; stage 3-4 of renal failure) **(chapter 2)** we found increased plasma TNFα and IL-6 along with increased SOCS3 in monocytes *vs.* increased SOCS1 in lymphocytes. This indicated that increased systemic inflammation is accompanied by increased SOCS expression and it affects monocytes and lymphocytes differentially. This later was confirmed by two sets of complimentary data. First, exposure of whole blood to a single cytokine (IL-6) resulted in differential responses in SOCS expression in monocytes and lymphocyte. Second, correlation studies indicated that monocytes SOCS3 was significantly correlated with markers of renal function (i.e. GFR and serum urea), while lymphocyte SOCS1 was significantly correlated with increased systolic blood pressure, plasma TNFα, and increased pulse wave velocity. Decreased renal failure, increased systolic blood pressure, inflammation, and pulse wave velocity are known risk factors of cardiovascular disease, and therefore, our data linked increased MNC SOCS to the known risk factors of CVD, indicating the potential of SOCS as a new intracellular marker for CVD in renal patients (113).

In end stage renal disease (stage 3) additional factors (e.g. dialysate and contact with dialysis membrane) may cause further activation of MNC and increased inflammation (116). In fact, previous studies have pointed to a state of MNC dysfunction along with increased activation of MNC in ESRD (8, 22). In line with previous studies we found increased plasma levels of TNFα, IL-6 and CRP in ESRD (110, 111). Moreover, we found increased SOCS1 gene expression in monocytes and lymphocytes, and CIS-1 gene expression in lymphocytes of ESRD patients. A significant correlation was also found between monocyte SOCS1 and plasma IL-6. Further analysis of the data whether dialysis was initiated or not and regarding dialysis modality, indicated a similarity in the profile of SOCS expression in MNC of non-dialysis ESRD patients and peritoneal dialysis patients. In both groups CIS-1 gene expression was significantly increased in lymphocytes and SOCS1 was also increased in lymphocytes although the expression of SOCS1 was only marginally significant in non-dialysis patients. In addition, a significant correlation was found between lymphocyte CIS-1 and plasma TNFα in non-dialysis patients **(chapter 3)**. Together, these data confirmed our previous conclusion that increased inflammation in renal patients is accompanied by increased SOCS expression in MNC. Furthermore, hemodialysis may have substantial effect on monocytes and lymphocyte activation and responsiveness, which is indicated by differential SOCS expression in MNC of hemodialysis patients comparing to non-dialysis and peritoneal dialysis.

Next, we were interested to understand how increased SOCS could affect cytokine signaling. In particular we were wondering whether SOCS could discriminate between pro-inflammatory and anti-inflammatory signals. In this regard, IL-6 was the most suitable candidate, as it increased in all patients in our study and has both pro and anti-inflammatory effects (95, 140). IL-6 is mostly increased together with other cytokines. Previous studies indicated that for instance TNFα and IL-1β are able to inhibit IL-6-induced STAT3 activation (63, 142). In this thesis we studied the interaction between IFNγ and IL-6 **(chapter 4 and 5)**. IFNγ is a pro-inflammatory and pro-atherogenic cytokine that mainly signals through STAT1 and induces SOCS in different cell types (112). The idea was to induce SOCS within the cells in prior to IL-6 stimulation and test how pro-inflammatory and anti-inflammatory signals are affected. We explored this interaction in endothelial cells, which are important in initiation and progression of atherosclerosis. In addition, endothelial dysfunction has been demonstrated in CRF and suggested to be a cause of increased atherosclerosis in CRF (34-36). Our data indicated that IFNγ pretreatment of EC led to inhibition of IL-6-induced STAT3 activation in a SOCS3-dependent fashion **(chapter 4)**. STAT3 is the main transcription factor in IL-6 signaling and carries the most well characterized effects of IL-6 including its anti-inflammatory effects and acute phase response induction in the liver (75, 195). Using gene expression profiling we characterized the transcriptome induced by IL-6 in EC in the presence or absence of IFNγ **(chapter 5)**. Our data indicated that IL-6, in contrast to IFNγ, induces relatively a small number of genes with both pro- as well as anti-inflammatory effects. The presence of IFNγ, however, inhibited some protective IL-6 target genes including Id-1, Pim-1, and Myc and simultaneously some inflammatory genes were increased. These sets of genes were IFNγ target genes (e.g. CCL8 and CXCL11) or genes that were induced by both cytokines (e.g. NLF-2 and MCP-1). In particular, the expression of some chemokines (CCL8, CXCL11, MCP-2, and CXCL2) was increased. Chemokines could have atherogenic effects by attracting the MNC through the endothelial cell layer (156). Furthermore, our data indicate that IL-6 as well as IFNγ induced gene in STAT-dependent and independent fashions. It is known that IL-6 can activate both STAT1 and STAT3 in different cell types (75, 194). As this two transcription factors have apposing role (STAT3 anti-inflammatory and STAT1 pro-inflammatory) (150, 151) we suggested that STAT1 might carry some pro-inflammatory effects of IL-6 **(chapter 6)**. To test this idea, in a pilot study we treated the wild type and $STAT1^{-/-}$ mice with IL-6 and studied

the expression of some acute phase genes (e.g. SAA-1) in the liver. Our data indicated that IL-6-induced SAA-1 expression was substantially decreased in STAT1$^{-/-}$ mice, indicating the importance of STAT1 as a part of IL-6 signaling. However, this work needs to be expanded.

Discussion and perspectives

1- Increased SOCS expression in monocytes or lymphocytes of CRF patients as a new marker of atherosclerosis.

This study is the first attempt to characterize the pattern of SOCS expression in circulating MNC in renal patients. Our data indicated that progressive loss of renal function is accompanied by increased inflammation and this, in turn, by increased distinct SOCS in distinct subtype of circulating MNC. Correlations were also found between increased SOCS expression and some other known risk factors of CVD. This led us to suggest that SOCS could be a new intracellular marker of CVD in renal patients. However, our results and the value of SOCS as a new CVD marker need to be confirmed in a bigger cohort. In addition it should be demonstrated that increased SOCS expression in MNC is accompanied by atherosclerosis development in CRF, For instance, whether increased (distinct) SOCS in MNC is accompanied by increased cell surface adhesion molecules, and or endothelial cell dysfunction, that is involved in atherosclerosis development.

2- Inhibition of SOCS3 and induction of SOCS1 in monocytes of CRF patients could delay or limit atherosclerosis.

Our data are not suitable to test a causal link between SOCS expression in MNC and development of atherosclerosis. It is however known that monocytes in ESRD have premature macrophage characteristic with increased cell surface adhesion molecules (29). It is, therefore, interesting to study the correlations between increased SOCS expression and cell surface adhesion molecules in CRF. In chapter 5, we indicated that interaction between IFNγ and IL-6 leads to increased expression of chemokines along with increased SOCS expression in endothelial cells. Interestingly, we indicated increased monocyte SOCS3 and plasma IL-6 and TNFα in CKD patients. The question is whether increased monocyte SOCS3 is accompanied by increased cell surface adhesion molecules.

There is yet no information available regarding the presence of SOCS in human atherosclerosis lesions. However, in ApoE$^{-/-}$ mice the presence of SOCS1 and SOCS3 co-located with macrophages in atherosclerosis lesions has been demonstrated (61). This is important since macrophages might have a central role in progression of atherosclerosis. In accord, deletion of SOCS3 from macrophages led to decreased atherosclerosis in ApoE$^{-/-}$ mice (62). These data linked SOCS with atherosclerosis, and indicated that manipulation of a distinct SOCS in monocytes/macrophages could have therapeutic values. This might also suggest that increased SOCS3 in monocytes of CKD patients could have pro-atherogenic effects **(chapter 2)**. Therefore, molecules that could diminish SOCS3 expression in monocytes might have potential therapeutic indications. Moreover, SOCS1 is an interesting molecule for therapeutic intentions. In contrast to SOCS3, that has both pro-and anti-inflammatory effects (59, 60), SOCS1 expression is mostly related to dampening of the inflammatory process (45, 49). In atherosclerosis, increased SOCS1 in monocytes could have also potential value in limitation or prevention of inflammation. This is based on the fact that SOCS1 strongly inhibits IFNγ, the classic macrophage activator, and also other inflammatory cytokines (49). This idea could be tested by breading the SOCS1 transgenic mice with ApoE$^{-/-}$ mice, and studying the atherosclerosis development in response to IFNγ treatment.

SOCS1 expression was increased in lymphocytes of both CKD and ESRD patients **(chapters 2-3)**. SOCS1 expression in T-cell could shift T-cell differentiation towards Th-1, which might have atherogenic effects (as atherosclerosis is a Th-1-type inflammatory disease) (53). Nevertheless, increased SOCS1 could inhibit lymphocyte responses to several inflammatory cytokines (49). The net protective or damaging effect of increased SOCS1 in lymphocytes needs, therefore, further investigation. CIS-1 was increased in lymphocytes of non-dialysis and peritoneal dialysis but not in hemodialysis and CKD patients **(chapter 2-3)**. CIS-1 seems to be necessary for survival of activated T-cells (123). It is also reported that T-cell count is decreased in hemodialysis patients (124). It is therefore, interesting to study whether there is a link between unchanged CIS-1 expression in lymphocytes of hemodialysis and decreased count number of T-cells.

We indicated that there are differences in the pattern of SOCS expression in CKD patients comparing to the ESRD **(chapters 2-3)**. However, this is a cross-sectioned study and we did not follow the patients over the time. It is clear that the chance of CVD death in CKD

patients is 5-10 time more than the chance of getting the ESRD stage (14). The question is whether there is a difference regarding the SOCS expression in their MNC between patients how reach the ESRD stage and others who died, and whether entering the ESRD is accompanied by changes in the pattern of SOCS expression in MNC. In CKD we found increased monocyte SOCS3, which as discussed could have potential atherogenic effects. In ESRD, SOCS1 was slightly increased in monocytes, which potentially could have anti-inflammatory effects in monocytes. The question is whether SOCS1 was increased in pre-dialysis stages, or there is a shift from SOCS3 to SOCS1 expression in monocytes. This could suggest that manipulation of SOCS expression in monocytes in earlier stages of renal failure could have prevention benefits.

At last we should admit that we can not ascertain whether increased SOCS expression in CKD and/or ESRD is in proportion to increased systemic inflammation. It is possible that increased SOCS expression in MNC is capable of inhibiting the protective signals (which are in minority) through Jak/STAT, while they can not counteract the pro-inflammatory signals (which are in majority) through Jak/STAT.

3- Both STAT1 and STAT3 and other transcription factor(s) are needed to cover the whole gene induction activity of IL-6 and IFNγ

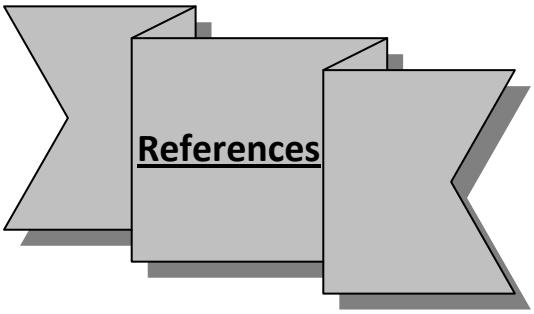
Our *in vivo* data indicated increased SOCS expression, however, evidences regarding how SOCS could affect cell response to other cytokines were provided by our *in vitro* studies.

As IL-6 is increased in CRF patients **(chapter 2-3)** and it mainly signals through the Jak/STAT pathway, we were interested to know what kind of responses are initiated in cells exposed to IL-6 and how increased SOCS by other cytokines could affect IL-6 signaling. The intention of our in vitro studies was to answer the following questions: a) since IL-6 has both anti- and pro-inflammatory effects, does SOCS equally modulate those effects? B) IL-6 signals predominantly through STAT3, however it activates STAT1 in different cell types. As STAT1 and STAT3 have opposing effects, could STAT1 carry some pro-inflammatory effects of IL-6?

Pretreatment of EC with IFNγ resulted in induction of both SOCS1 and SOCS3 **(chapter 4)**. This, in turn diminished IL-6-induced STAT3 activation in a SOCS3-dependent manner. Using gene array we studied the effect of this interaction on the genes induced by IL-6 **(chapter 5)**. Our data indicated that IL-6 alone induced a few pro- as well as anti-

inflammatory genes. The new challenge is to identify whether anti-inflammatory effects of IL-6 are related to *de novo* gene induction or suppression of inflammatory genes induced by other stimuli. Furthermore, inhibition of IL-6-induced STAT3 activation led to inhibition of some proliferative and protective STAT3-depenent genes including Pim-1, Id-1 and Myc. Simultaneously, other genes, which might not be STAT3-dependent were increased, in particular the expression of some chemokines including MCP-1, CXCL2, CCL8 and CXCL11 were increased. It is now the question whether increased expression of chemokines could substantially increase adhesiveness of endothelial cells and attraction and migration of MNC through endothelial cell layer.

Previous studies indicated that the expression of some chemokines (e.g. CCL8 and CXCL11) upon IFNγ is STAT1-dependent (157, 186), while the others (e.g. MCP-1) is STAT1-independent (157). The expression of these genes was increased in the presence of IL-6, indicating that IL-6 might potentiate the expression of some genes through STAT1. The classic idea is that STAT1 carries the well characterized effects of IFNγ (148) and STAT3 of IL-6 (149). However, our data indicate that other transcription factors are needed to cover the whole gene induction activities of these cytokines. This is also a challenging area for follow up studies. We have already taken the first step to unravel this enigma. In a pilot study using $STAT1^{-/-}$ model we indicated that at least induction of some acute phase genes (which is believed to be STAT3-dependent) is substantially decreased in $STAT1^{-/-}$ mice upon IL-6 treatment **(chapter 6)**.


References

References

1. Stenvinkel, P. 2002. Inflammation in end-stage renal failure: could it be treated? *Nephrol Dial Transplant* 17 Suppl 8:33-38; discussion 40.
2. Levey, A.S., Beto, J.A., Coronado, B.E., Eknoyan, G., Foley, R.N., Kasiske, B.L., Klag, M.J., Mailloux, L.U., Manske, C.L., Meyer, K.B., et al. 1998. Controlling the epidemic of cardiovascular disease in chronic renal disease: what do we know? What do we need to learn? Where do we go from here? National Kidney Foundation Task Force on Cardiovascular Disease. *Am J Kidney Dis* 32:853-906.
3. Stenvinkel, P., Pecoits-Filho, R., and Lindholm, B. 2003. Coronary artery disease in end-stage renal disease: no longer a simple plumbing problem. *J Am Soc Nephrol* 14:1927-1939.
4. Galle, J., Quaschning, T., Seibold, S., and Wanner, C. 2003. Endothelial dysfunction and inflammation: what is the link? *Kidney Int Suppl*:S45-49.
5. Mathur, S., Devaraj, S., and Jialal, I. 2002. Accelerated atherosclerosis, dyslipidemia, and oxidative stress in end-stage renal disease. *Curr Opin Nephrol Hypertens* 11:141-147.
6. Libby, P. 2002. Inflammation in atherosclerosis. *Nature* 420:868-874.
7. Bolton, C.H., Downs, L.G., Victory, J.G., Dwight, J.F., Tomson, C.R., Mackness, M.I., and Pinkney, J.H. 2001. Endothelial dysfunction in chronic renal failure: roles of lipoprotein oxidation and pro-inflammatory cytokines. *Nephrol Dial Transplant* 16:1189-1197.
8. Girndt, M., Sester, M., Sester, U., Kaul, H., and Kohler, H. 2001. Molecular aspects of T- and B-cell function in uremia. *Kidney Int Suppl* 78:S206-211.
9. Thambyrajah, J., Landray, M.J., McGlynn, F.J., Jones, H.J., Wheeler, D.C., and Townend, J.N. 2000. Abnormalities of endothelial function in patients with predialysis renal failure. *Heart* 83:205-209.
10. Gris, J.C., Branger, B., Vecina, F., al Sabadani, B., Fourcade, J., and Schved, J.F. 1994. Increased cardiovascular risk factors and features of endothelial activation and dysfunction in dialyzed uremic patients. *Kidney Int* 46:807-813.
11. Hansson, G.K., Robertson, A.K., and Soderberg-Naucler, C. 2006. Inflammation and atherosclerosis. *Annu Rev Pathol* 1:297-329.
12. Kwak, B.R., Mulhaupt, F., and Mach, F. 2003. Atherosclerosis: anti-inflammatory and immunomodulatory activities of statins. *Autoimmun Rev* 2:332-338.
13. Go, A.S., Chertow, G.M., Fan, D., McCulloch, C.E., and Hsu, C.Y. 2004. Chronic kidney disease and the risks of death, cardiovascular events, and hospitalization. *N Engl J Med* 351:1296-1305.
14. Collins, A.J., Li, S., Gilbertson, D.T., Liu, J., Chen, S.C., and Herzog, C.A. 2003. Chronic kidney disease and cardiovascular disease in the Medicare population. *Kidney Int Suppl*:S24-31.
15. Manjunath, G., Tighiouart, H., Ibrahim, H., MacLeod, B., Salem, D.N., Griffith, J.L., Coresh, J., Levey, A.S., and Sarnak, M.J. 2003. Level of kidney function as a risk factor for atherosclerotic cardiovascular outcomes in the community. *J Am Coll Cardiol* 41:47-55.
16. Longenecker, J.C., Coresh, J., Powe, N.R., Levey, A.S., Fink, N.E., Martin, A., and Klag, M.J. 2002. Traditional cardiovascular disease risk factors in dialysis patients

compared with the general population: the CHOICE Study. *J Am Soc Nephrol* 13:1918-1927.

17. Yao, Q., Pecoits-Filho, R., Lindholm, B., and Stenvinkel, P. 2004. Traditional and non-traditional risk factors as contributors to atherosclerotic cardiovascular disease in end-stage renal disease. *Scand J Urol Nephrol* 38:405-416.
18. Stenvinkel, P., Heimburger, O., Paultre, F., Diczfalusy, U., Wang, T., Berglund, L., and Jogestrand, T. 1999. Strong association between malnutrition, inflammation, and atherosclerosis in chronic renal failure. *Kidney Int* 55:1899-1911.
19. Herbelin, A., Urena, P., Nguyen, A.T., Zingraff, J., and Descamps-Latscha, B. 1991. Elevated circulating levels of interleukin-6 in patients with chronic renal failure. *Kidney Int* 39:954-960.
20. Pereira, B.J., Shapiro, L., King, A.J., Falagas, M.E., Strom, J.A., and Dinarello, C.A. 1994. Plasma levels of IL-1 beta, TNF alpha and their specific inhibitors in undialyzed chronic renal failure, CAPD and hemodialysis patients. *Kidney Int* 45:890-896.
21. Nakanishi, I., Moutabarrik, A., Okada, N., Kitamura, E., Hayashi, A., Syouji, T., Namiki, M., Ishibashi, M., Zaid, D., and Tsubakihara, Y. 1994. Interleukin-8 in chronic renal failure and dialysis patients. *Nephrol Dial Transplant* 9:1435-1442.
22. Girndt, M., Kohler, H., Schiedhelm-Weick, E., Schlaak, J.F., Meyer zum Buschenfelde, K.H., and Fleischer, B. 1995. Production of interleukin-6, tumor necrosis factor alpha and interleukin-10 in vitro correlates with the clinical immune defect in chronic hemodialysis patients. *Kidney Int* 47:559-565.
23. Litjens, N.H., van Druningen, C.J., and Betjes, M.G. 2006. Progressive loss of renal function is associated with activation and depletion of naive T lymphocytes. *Clin Immunol* 118:83-91.
24. Raska, K., Jr., Raskova, J., Shea, S.M., Frankel, R.M., Wood, R.H., Lifter, J., Ghobrial, I., Eisinger, R.P., and Homer, L. 1983. T cell subsets and cellular immunity in end-stage renal disease. *Am J Med* 75:734-740.
25. Meuer, S.C., Hauer, M., Kurz, P., Meyer zum Buschenfelde, K.H., and Kohler, H. 1987. Selective blockade of the antigen-receptor-mediated pathway of T cell activation in patients with impaired primary immune responses. *J Clin Invest* 80:743-749.
26. Beaurain, G., Naret, C., Marcon, L., Grateau, G., Drueke, T., Urena, P., Nelson, D.L., Bach, J.F., and Chatenoud, L. 1989. In vivo T cell preactivation in chronic uremic hemodialyzed and non-hemodialyzed patients. *Kidney Int* 36:636-644.
27. Dinarello, C.A. 1992. Cytokines: agents provocateurs in hemodialysis? *Kidney Int* 41:683-694.
28. Witko-Sarsat, V., Gausson, V., and Descamps-Latscha, B. 2003. Are advanced oxidation protein products potential uremic toxins? *Kidney Int Suppl*:S11-14.
29. Brauner, A., Lu, Y., Hallden, G., Hylander, B., and Lundahl, J. 1998. Difference in the blood monocyte phenotype between uremic patients and healthy controls: its relation to monocyte differentiation into macrophages in the peritoneal cavity. *Inflammation* 22:55-66.
30. Matsumoto, A., Seki, Y., Kubo, M., Ohtsuka, S., Suzuki, A., Hayashi, I., Tsuji, K., Nakahata, T., Okabe, M., Yamada, S., et al. 1999. Suppression of STAT5 functions in liver, mammary glands, and T cells in cytokine-inducible SH2-containing protein 1 transgenic mice. *Mol Cell Biol* 19:6396-6407.

31. Nitta, K., Akiba, T., Kawashima, A., Kimata, N., Miwa, N., Nishida, E., Uchida, K., Honda, K., Yumura, W., and Nihei, H. 2002. Characterization of TH1/TH2 profile in uremic patients. *Nephron* 91:492-495.
32. Sester, U., Sester, M., Hauk, M., Kaul, H., Kohler, H., and Girndt, M. 2000. T-cell activation follows Th1 rather than Th2 pattern in haemodialysis patients. *Nephrol Dial Transplant* 15:1217-1223.
33. Stenvinkel, P. 2001. Endothelial dysfunction and inflammation-is there a link? *Nephrol Dial Transplant* 16:1968-1971.
34. Annuk, M., Lind, L., Linde, T., and Fellstrom, B. 2001. Impaired endothelium-dependent vasodilatation in renal failure in humans. *Nephrol Dial Transplant* 16:302-306.
35. Miyazaki, H., Matsuoka, H., Itabe, H., Usui, M., Ueda, S., Okuda, S., and Imaizumi, T. 2000. Hemodialysis impairs endothelial function via oxidative stress: effects of vitamin E-coated dialyzer. *Circulation* 101:1002-1006.
36. van Guldener, C., Janssen, M.J., Lambert, J., Steyn, M., Donker, A.J., and Stehouwer, C.D. 1998. Endothelium-dependent vasodilatation is impaired in peritoneal dialysis patients. *Nephrol Dial Transplant* 13:1782-1786.
37. Bevilacqua, M.P. 1993. Endothelial-leukocyte adhesion molecules. *Annu Rev Immunol* 11:767-804.
38. Sinisalo, J., Paronen, J., Mattila, K.J., Syrjala, M., Alfthan, G., Palosuo, T., Nieminen, M.S., and Vaarala, O. 2000. Relation of inflammation to vascular function in patients with coronary heart disease. *Atherosclerosis* 149:403-411.
39. Kisseleva, T., Bhattacharya, S., Braunstein, J., and Schindler, C.W. 2002. Signaling through the JAK/STAT pathway, recent advances and future challenges. *Gene* 285:1-24.
40. Krebs, D.L., and Hilton, D.J. 2001. SOCS proteins: negative regulators of cytokine signaling. *Stem Cells* 19:378-387.
41. Grote, K., Luchtefeld, M., and Schieffer, B. 2005. JANUS under stress--role of JAK/STAT signaling pathway in vascular diseases. *Vascul Pharmacol* 43:357-363.
42. Alexander, W.S., Starr, R., Metcalf, D., Nicholson, S.E., Farley, A., Elefanty, A.G., Brysha, M., Kile, B.T., Richardson, R., Baca, M., et al. 1999. Suppressors of cytokine signaling (SOCS): negative regulators of signal transduction. *J Leukoc Biol* 66:588-592.
43. Young, H.A., Klinman, D.M., Reynolds, D.A., Grzegorzewski, K.J., Nii, A., Ward, J.M., Winkler-Pickett, R.T., Ortaldo, J.R., Kenny, J.J., and Komschlies, K.L. 1997. Bone marrow and thymus expression of interferon-gamma results in severe B-cell lineage reduction, T-cell lineage alterations, and hematopoietic progenitor deficiencies. *Blood* 89:583-595.
44. Starr, R., and Hilton, D.J. 1999. Negative regulation of the JAK/STAT pathway. *Bioessays* 21:47-52.
45. Chen, W., Daines, M.O., and Khurana Hershey, G.K. 2004. Turning off signal transducer and activator of transcription (STAT): the negative regulation of STAT signaling. *J Allergy Clin Immunol* 114:476-489; quiz 490.
46. Yoshimura, A., Ohkubo, T., Kiguchi, T., Jenkins, N.A., Gilbert, D.J., Copeland, N.G., Hara, T., and Miyajima, A. 1995. A novel cytokine-inducible gene CIS encodes an SH2-containing protein that binds to tyrosine-phosphorylated interleukin 3 and erythropoietin receptors. *Embo J* 14:2816-2826.

47. Naka, T., Narazaki, M., Hirata, M., Matsumoto, T., Minamoto, S., Aono, A., Nishimoto, N., Kajita, T., Taga, T., Yoshizaki, K., et al. 1997. Structure and function of a new STAT-induced STAT inhibitor. *Nature* 387:924-929.
48. Starr, R., Willson, T.A., Viney, E.M., Murray, L.J., Rayner, J.R., Jenkins, B.J., Gonda, T.J., Alexander, W.S., Metcalf, D., Nicola, N.A., et al. 1997. A family of cytokine-inducible inhibitors of signalling. *Nature* 387:917-921.
49. Fujimoto, M., Tsutsui, H., Xinshou, O., Tokumoto, M., Watanabe, D., Shima, Y., Yoshimoto, T., Hirakata, H., Kawase, I., Nakanishi, K., et al. 2004. Inadequate induction of suppressor of cytokine signaling-1 causes systemic autoimmune diseases. *Int Immunol* 16:303-314.
50. Chen, Y., Chong, M.M., Darwiche, R., Thomas, H.E., and Kay, T.W. 2004. Severe pancreatitis with exocrine destruction and increased islet neogenesis in mice with suppressor of cytokine signaling-1 deficiency. *Am J Pathol* 165:913-921.
51. Hilton, D.J., Richardson, R.T., Alexander, W.S., Viney, E.M., Willson, T.A., Sprigg, N.S., Starr, R., Nicholson, S.E., Metcalf, D., and Nicola, N.A. 1998. Twenty proteins containing a C-terminal SOCS box form five structural classes. *Proc Natl Acad Sci U S A* 95:114-119.
52. Yasukawa, H., Misawa, H., Sakamoto, H., Masuhara, M., Sasaki, A., Wakioka, T., Ohtsuka, S., Imaizumi, T., Matsuda, T., Ihle, J.N., et al. 1999. The JAK-binding protein JAB inhibits Janus tyrosine kinase activity through binding in the activation loop. *Embo J* 18:1309-1320.
53. Tang, J., and Raines, E.W. 2005. Are suppressors of cytokine signaling proteins recently identified in atherosclerosis possible therapeutic targets? *Trends Cardiovasc Med* 15:243-249.
54. Ito, S., Ansari, P., Sakatsume, M., Dickensheets, H., Vazquez, N., Donnelly, R.P., Larner, A.C., and Finbloom, D.S. 1999. Interleukin-10 inhibits expression of both interferon alpha- and interferon gamma- induced genes by suppressing tyrosine phosphorylation of STAT1. *Blood* 93:1456-1463.
55. Ram, P.A., and Waxman, D.J. 2000. Role of the cytokine-inducible SH2 protein CIS in desensitization of STAT5b signaling by continuous growth hormone. *J Biol Chem* 275:39487-39496.
56. Zhang, J.G., Farley, A., Nicholson, S.E., Willson, T.A., Zugaro, L.M., Simpson, R.J., Moritz, R.L., Cary, D., Richardson, R., Hausmann, G., et al. 1999. The conserved SOCS box motif in suppressors of cytokine signaling binds to elongins B and C and may couple bound proteins to proteasomal degradation. *Proc Natl Acad Sci U S A* 96:2071-2076.
57. Favre, H., Benhamou, A., Finidori, J., Kelly, P.A., and Edery, M. 1999. Dual effects of suppressor of cytokine signaling (SOCS-2) on growth hormone signal transduction. *FEBS Lett* 453:63-66.
58. Farabegoli, F., Ceccarelli, C., Santini, D., and Taffurelli, M. 2005. Suppressor of cytokine signalling 2 (SOCS-2) expression in breast carcinoma. *J Clin Pathol* 58:1046-1050.
59. Shouda, T., Yoshida, T., Hanada, T., Wakioka, T., Oishi, M., Miyoshi, K., Komiya, S., Kosai, K., Hanakawa, Y., Hashimoto, K., et al. 2001. Induction of the cytokine signal regulator SOCS3/CIS3 as a therapeutic strategy for treating inflammatory arthritis. *J Clin Invest* 108:1781-1788.

60. Seki, Y., Inoue, H., Nagata, N., Hayashi, K., Fukuyama, S., Matsumoto, K., Komine, O., Hamano, S., Himeno, K., Inagaki-Ohara, K., et al. 2003. SOCS-3 regulates onset and maintenance of T(H)2-mediated allergic responses. *Nat Med* 9:1047-1054.
61. Tang, J., Kozaki, K., Farr, A.G., Martin, P.J., Lindahl, P., Betsholtz, C., and Raines, E.W. 2005. The absence of platelet-derived growth factor-B in circulating cells promotes immune and inflammatory responses in atherosclerosis-prone ApoE-/- mice. *Am J Pathol* 167:901-912.
62. Yamamoto T, Y.H., Koga M, et al. . 2007. Lacking the SOCS3 Gene in Macrophages Prevents the Development of Atherosclerosis in ApoE Deficient Mice *Circ J* 71:170.
63. Niemand, C., Nimmesgern, A., Haan, S., Fischer, P., Schaper, F., Rossaint, R., Heinrich, P.C., and Muller-Newen, G. 2003. Activation of STAT3 by IL-6 and IL-10 in primary human macrophages is differentially modulated by suppressor of cytokine signaling 3. *J Immunol* 170:3263-3272.
64. Alexander, W.S. 2002. Suppressors of cytokine signalling (SOCS) in the immune system. *Nat Rev Immunol* 2:410-416.
65. Ueki, K., Kadowaki, T., and Kahn, C.R. 2005. Role of suppressors of cytokine signaling SOCS-1 and SOCS-3 in hepatic steatosis and the metabolic syndrome. *Hepatol Res* 33:185-192.
66. Yasukawa, H., Yajima, T., Duplain, H., Iwatate, M., Kido, M., Hoshijima, M., Weitzman, M.D., Nakamura, T., Woodard, S., Xiong, D., et al. 2003. The suppressor of cytokine signaling-1 (SOCS1) is a novel therapeutic target for enterovirus-induced cardiac injury. *J Clin Invest* 111:469-478.
67. Egan, P.J., Lawlor, K.E., Alexander, W.S., and Wicks, I.P. 2003. Suppressor of cytokine signaling-1 regulates acute inflammatory arthritis and T cell activation. *J Clin Invest* 111:915-924.
68. Wong, P.K., Egan, P.J., Croker, B.A., O'Donnell, K., Sims, N.A., Drake, S., Kiu, H., McManus, E.J., Alexander, W.S., Roberts, A.W., et al. 2006. SOCS-3 negatively regulates innate and adaptive immune mechanisms in acute IL-1-dependent inflammatory arthritis. *J Clin Invest* 116:1571-1581.
69. Macdougall, I.C., and Cooper, A.C. 2002. Erythropoietin resistance: the role of inflammation and pro-inflammatory cytokines. *Nephrol Dial Transplant* 17 Suppl 11:39-43.
70. Jelkmann, W. 1998. Proinflammatory cytokines lowering erythropoietin production. *J Interferon Cytokine Res* 18:555-559.
71. Sun, D.F., Zheng, Z., Tummala, P., Oh, J., Schaefer, F., and Rabkin, R. 2004. Chronic uremia attenuates growth hormone-induced signal transduction in skeletal muscle. *J Am Soc Nephrol* 15:2630-2636.
72. Rabkin, R., Sun, D.F., Chen, Y., Tan, J., and Schaefer, F. 2005. Growth hormone resistance in uremia, a role for impaired JAK/STAT signaling. *Pediatr Nephrol* 20:313-318.
73. Sasaki, A., Yasukawa, H., Shouda, T., Kitamura, T., Dikic, I., and Yoshimura, A. 2000. CIS3/SOCS-3 suppresses erythropoietin (EPO) signaling by binding the EPO receptor and JAK2. *J Biol Chem* 275:29338-29347.
74. Yoshimura, A., Mori, H., Ohishi, M., Aki, D., and Hanada, T. 2003. Negative regulation of cytokine signaling influences inflammation. *Curr Opin Immunol* 15:704-708.

75. Alonzi, T., Maritano, D., Gorgoni, B., Rizzuto, G., Libert, C., and Poli, V. 2001. Essential role of STAT3 in the control of the acute-phase response as revealed by inducible gene inactivation [correction of activation] in the liver. *Mol Cell Biol* 21:1621-1632.
76. Roberts, M.A., Hare, D.L., Ratnaike, S., and Ierino, F.L. 2006. Cardiovascular biomarkers in CKD: pathophysiology and implications for clinical management of cardiac disease. *Am J Kidney Dis* 48:341-360.
77. Leskinen, Y., Groundstroem, K., Virtanen, V., Lehtimaki, T., Huhtala, H., and Saha, H. 2003. Risk factors for aortic atherosclerosis determined by transesophageal echocardiography in patients with CRF. *Am J Kidney Dis* 42:277-285.
78. Chan, C.M. 2005. Hyperlipidaemia in chronic kidney disease. *Ann Acad Med Singapore* 34:31-35.
79. Inagaki-Ohara, K., Hanada, T., and Yoshimura, A. 2003. Negative regulation of cytokine signaling and inflammatory diseases. *Curr Opin Pharmacol* 3:435-442.
80. Ihle, J.N. 1995. Cytokine receptor signalling. *Nature* 377:591-594.
81. Krebs, D.L., and Hilton, D.J. 2000. SOCS: physiological suppressors of cytokine signaling. *J Cell Sci* 113 (Pt 16):2813-2819.
82. Tan, J.C., and Rabkin, R. 2005. Suppressors of cytokine signaling in health and disease. *Pediatr Nephrol* 20:567-575.
83. Starr, R., Metcalf, D., Elefanty, A.G., Brysha, M., Willson, T.A., Nicola, N.A., Hilton, D.J., and Alexander, W.S. 1998. Liver degeneration and lymphoid deficiencies in mice lacking suppressor of cytokine signaling-1. *Proc Natl Acad Sci U S A* 95:14395-14399.
84. Marine, J.C., McKay, C., Wang, D., Topham, D.J., Parganas, E., Nakajima, H., Pendeville, H., Yasukawa, H., Sasaki, A., Yoshimura, A., et al. 1999. SOCS3 is essential in the regulation of fetal liver erythropoiesis. *Cell* 98:617-627.
85. Herbelin, A., Urena, P., Nguyen, A.T., Zingraff, J., and Descamps-Latscha, B. 1991. Influence of first and long-term dialysis on uraemia-associated increased basal production of interleukin-1 and tumour necrosis factor alpha by circulating monocytes. *Nephrol Dial Transplant* 6:349-357.
86. Rostaing, L., Borde, J.S., Hasle, C., Bories, P., Allal, A., Abbal, M., and Durand, D. 2001. Lack of effect of chronic hepatitis C virus infection on T-cell cytokine production in chronic hemodialysis patients. *Am J Nephrol* 21:194-199.
87. Tsirpanlis, G. 2007. Is Inflammation the Link between Atherosclerosis and Vascular Calcification in Chronic Kidney Disease? *Blood Purif* 25:179-182.
88. Carlos, T., Kovach, N., Schwartz, B., Rosa, M., Newman, B., Wayner, E., Benjamin, C., Osborn, L., Lobb, R., and Harlan, J. 1991. Human monocytes bind to two cytokine-induced adhesive ligands on cultured human endothelial cells: endothelial-leukocyte adhesion molecule-1 and vascular cell adhesion molecule-1. *Blood* 77:2266-2271.
89. Chomarat, P., Banchereau, J., Davoust, J., and Palucka, A.K. 2000. IL-6 switches the differentiation of monocytes from dendritic cells to macrophages. *Nat Immunol* 1:510-514.
90. Yoshimura, A., Nishinakamura, H., Matsumura, Y., and Hanada, T. 2005. Negative regulation of cytokine signaling and immune responses by SOCS proteins. *Arthritis Res Ther* 7:100-110.
91. Chong, M.M., Metcalf, D., Jamieson, E., Alexander, W.S., and Kay, T.W. 2005. Suppressor of cytokine signaling-1 in T cells and macrophages is critical for preventing lethal inflammation. *Blood* 106:1668-1675.

92. Vuurmans, T.J., Boer, P., and Koomans, H.A. 2003. Effects of endothelin-1 and endothelin-1 receptor blockade on cardiac output, aortic pressure, and pulse wave velocity in humans. *Hypertension* 41:1253-1258.
93. Flo, R.W., Naess, A., Lund-Johansen, F., Maehle, B.O., Sjursen, H., Lehmann, V., and Solberg, C.O. 1991. Negative selection of human monocytes using magnetic particles covered by anti-lymphocyte antibodies. *J Immunol Methods* 137:89-94.
94. Schumacher HR, Garvin D.F., Triplett D.A., 1984. Introduction to laboratory hematology and hematopathology, first ed. Alan R. Liss. Inc. New York, NY, pp. 9-107.
95. Stenvinkel, P., Ketteler, M., Johnson, R.J., Lindholm, B., Pecoits-Filho, R., Riella, M., Heimburger, O., Cederholm, T., and Girndt, M. 2005. IL-10, IL-6, and TNF-alpha: central factors in the altered cytokine network of uremia--the good, the bad, and the ugly. *Kidney Int* 67:1216-1233.
96. Briet, M., Bozec, E., Laurent, S., Fassot, C., London, G.M., Jacquot, C., Froissart, M., Houillier, P., and Boutouyrie, P. 2006. Arterial stiffness and enlargement in mild-to-moderate chronic kidney disease. *Kidney Int* 69:350-357.
97. Blacher, J., Guerin, A.P., Pannier, B., Marchais, S.J., Safar, M.E., and London, G.M. 1999. Impact of aortic stiffness on survival in end-stage renal disease. *Circulation* 99:2434-2439.
98. Ashman, N., Macey, M.G., Fan, S.L., Azam, U., and Yaqoob, M.M. 2003. Increased platelet-monocyte aggregates and cardiovascular disease in end-stage renal failure patients. *Nephrol Dial Transplant* 18:2088-2096.
99. Hernandez-Vargas, P., Lopez-Franco, O., Sanjuan, G., Ruperez, M., Ortiz-Munoz, G., Suzuki, Y., Aguado-Roncero, P., Perez-Tejerizo, G., Blanco, J., Egido, J., et al. 2005. Suppressors of cytokine signaling regulate angiotensin II-activated Janus kinase-signal transducers and activators of transcription pathway in renal cells. *J Am Soc Nephrol* 16:1673-1683.
100. Alexander, W.S., and Hilton, D.J. 2004. The role of suppressors of cytokine signaling (SOCS) proteins in regulation of the immune response. *Annu Rev Immunol* 22:503-529.
101. Croker, B.A., Krebs, D.L., Zhang, J.G., Wormald, S., Willson, T.A., Stanley, E.G., Robb, L., Greenhalgh, C.J., Forster, I., Clausen, B.E., et al. 2003. SOCS3 negatively regulates IL-6 signaling in vivo. *Nat Immunol* 4:540-545.
102. Rui, L., Yuan, M., Frantz, D., Shoelson, S., and White, M.F. 2002. SOCS-1 and SOCS-3 block insulin signaling by ubiquitin-mediated degradation of IRS1 and IRS2. *J Biol Chem* 277:42394-42398.
103. Adams, T.E., Hansen, J.A., Starr, R., Nicola, N.A., Hilton, D.J., and Billestrup, N. 1998. Growth hormone preferentially induces the rapid, transient expression of SOCS-3, a novel inhibitor of cytokine receptor signaling. *J Biol Chem* 273:1285-1287.
104. Qasimi, P., Ming-Lum, A., Ghanipour, A., Ong, C.J., Cox, M.E., Ihle, J., Cacalano, N., Yoshimura, A., and Mui, A.L. 2006. Divergent mechanisms utilized by SOCS3 to mediate interleukin-10 inhibition of tumor necrosis factor alpha and nitric oxide production by macrophages. *J Biol Chem* 281:6316-6324.
105. Huang, K.C., Chen, C.W., Chen, J.C., and Lin, W.W. 2003. Statins induce suppressor of cytokine signaling-3 in macrophages. *FEBS Lett* 555:385-389.

106. Robertson, A.K., and Hansson, G.K. 2006. T cells in atherogenesis: for better or for worse? *Arterioscler Thromb Vasc Biol* 26:2421-2432.
107. Hansson, G.K. 2005. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med* 352:1685-1695.
108. Alexander, W.S., Starr, R., Fenner, J.E., Scott, C.L., Handman, E., Sprigg, N.S., Corbin, J.E., Cornish, A.L., Darwiche, R., Owczarek, C.M., et al. 1999. SOCS1 is a critical inhibitor of interferon gamma signaling and prevents the potentially fatal neonatal actions of this cytokine. *Cell* 98:597-608.
109. Egwuagu, C.E., Yu, C.R., Zhang, M., Mahdi, R.M., Kim, S.J., and Gery, I. 2002. Suppressors of cytokine signaling proteins are differentially expressed in Th1 and Th2 cells: implications for Th cell lineage commitment and maintenance. *J Immunol* 168:3181-3187.
110. Malaponte, G., Bevelacqua, V., Fatuzzo, P., Rapisarda, F., Emmanuele, G., Travali, S., and Mazzarino, M.C. 2002. IL-1beta, TNF-alpha and IL-6 release from monocytes in haemodialysis patients in relation to dialytic age. *Nephrol Dial Transplant* 17:1964-1970.
111. Wong, C.K., Szeto, C.C., Chan, M.H., Leung, C.B., Li, P.K., and Lam, C.W. 2007. Elevation of pro-inflammatory cytokines, C-reactive protein and cardiac troponin T in chronic renal failure patients on dialysis. *Immunol Invest* 36:47-57.
112. Leon, M.L., and Zuckerman, S.H. 2005. Gamma interferon: a central mediator in atherosclerosis. *Inflamm Res* 54:395-411.
113. Rastmanesh, M.M., Bluyssen, H.A., Joles, J.A., Boer, P., Willekes, N., and Braam, B. 2008. Increased expression of SOCS3 in monocytes and SOCS1 in lymphocytes correlates with progressive loss of renal function and cardiovascular risk factors in chronic kidney disease. *Eur J Pharmacol* 593:99-104.
114. Attia, D.M., Ni, Z.N., Boer, P., Attia, M.A., Goldschmeding, R., Koomans, H.A., Vaziri, N.D., and Joles, J.A. 2002. Proteinuria is preceded by decreased nitric oxide synthesis and prevented by a NO donor in cholesterol-fed rats. *Kidney Int* 61:1776-1787.
115. Dhondt, A., Vanholder, R., Van Biesen, W., and Lameire, N. 2000. The removal of uremic toxins. *Kidney Int Suppl* 76:S47-59.
116. Martin-Malo, A., Carracedo, J., Ramirez, R., Rodriguez-Benot, A., Soriano, S., Rodriguez, M., and Aljama, P. 2000. Effect of uremia and dialysis modality on mononuclear cell apoptosis. *J Am Soc Nephrol* 11:936-942.
117. Menegatti, E., Rossi, D., Chiara, M., Alpa, M., Sena, L.M., and Roccatello, D. 2002. Cytokine release pathway in mononuclear cells stimulated in vitro by dialysis membranes. *Am J Nephrol* 22:509-514.
118. Walker, R.J., Sutherland, W.H., and De Jong, S.A. 2004. Effect of changing from a cellulose acetate to a polysulphone dialysis membrane on protein oxidation and inflammation markers. *Clin Nephrol* 61:198-206.
119. Wanner, C., Bahner, U., Mattern, R., Lang, D., and Passlick-Deetjen, J. 2004. Effect of dialysis flux and membrane material on dyslipidaemia and inflammation in haemodialysis patients. *Nephrol Dial Transplant* 19:2570-2575.
120. Tedgui, A., and Mallat, Z. 2006. Cytokines in atherosclerosis: pathogenic and regulatory pathways. *Physiol Rev* 86:515-581.

121. Klinger, J., Enriquez, J., Arturo, J.A., Delgado, M., Avila, G., and Ceballos, O. 2002. Cytokines and peritonitis in continuous ambulatory peritoneal dialysis: immunodeviation and immunodeficiency. *Adv Perit Dial* 18:170-176.
122. Meier, P., Dayer, E., Blanc, E., and Wauters, J.P. 2002. Early T cell activation correlates with expression of apoptosis markers in patients with end-stage renal disease. *J Am Soc Nephrol* 13:204-212.
123. Li, S., Chen, S., Xu, X., Sundstedt, A., Paulsson, K.M., Anderson, P., Karlsson, S., Sjogren, H.O., and Wang, P. 2000. Cytokine-induced Src homology 2 protein (CIS) promotes T cell receptor-mediated proliferation and prolongs survival of activated T cells. *J Exp Med* 191:985-994.
124. Moser, B., Roth, G., Brunner, M., Lilaj, T., Deicher, R., Wolner, E., Kovarik, J., Boltz-Nitulescu, G., Vychytil, A., and Ankersmit, H.J. 2003. Aberrant T cell activation and heightened apoptotic turnover in end-stage renal failure patients: a comparative evaluation between non-dialysis, haemodialysis, and peritoneal dialysis. *Biochem Biophys Res Commun* 308:581-585.
125. Cooper, A.C., Breen, C.P., Vyas, B., Ochola, J., Kemeny, D.M., and Macdougall, I.C. 2003. Poor response to recombinant erythropoietin is associated with loss of T-lymphocyte CD28 expression and altered interleukin-10 production. *Nephrol Dial Transplant* 18:133-140.
126. Fujimoto, M., Naka, T., Nakagawa, R., Kawazoe, Y., Morita, Y., Tateishi, A., Okumura, K., Narazaki, M., and Kishimoto, T. 2000. Defective thymocyte development and perturbed homeostasis of T cells in STAT-induced STAT inhibitor-1/suppressors of cytokine signaling-1 transgenic mice. *J Immunol* 165:1799-1806.
127. Novak, U., Marks, D., Nicholson, S.E., Hilton, D., and Paradiso, L. 1999. Differential ability of SOCS proteins to regulate IL-6 and CSF-1 induced macrophage differentiation. *Growth Factors* 16:305-314.
128. Wesemann, D.R., Dong, Y., O'Keefe, G.M., Nguyen, V.T., and Benveniste, E.N. 2002. Suppressor of cytokine signaling 1 inhibits cytokine induction of CD40 expression in macrophages. *J Immunol* 169:2354-2360.
129. Heine, G.H., Ulrich, C., Seibert, E., Seiler, S., Marell, J., Reichart, B., Krause, M., Schlitt, A., Kohler, H., and Girndt, M. 2008. CD14(++)CD16+ monocytes but not total monocyte numbers predict cardiovascular events in dialysis patients. *Kidney Int* 73:622-629.
130. Goicoechea, M., Martin, J., de Sequera, P., Quiroga, J.A., Ortiz, A., Carreno, V., and Caramelo, C. 1998. Role of cytokines in the response to erythropoietin in hemodialysis patients. *Kidney Int* 54:1337-1343.
131. van der Putten, K., Braam, B., Jie, K.E., and Gaillard, C.A. 2008. Mechanisms of Disease: erythropoietin resistance in patients with both heart and kidney failure. *Nat Clin Pract Nephrol* 4:47-57.
132. Wollert, K.C., and Drexler, H. 2001. The role of interleukin-6 in the failing heart. *Heart Fail Rev* 6:95-103.
133. Stenvinkel, P., Heimburger, O., and Jogestrand, T. 2002. Elevated interleukin-6 predicts progressive carotid artery atherosclerosis in dialysis patients: association with Chlamydia pneumoniae seropositivity. *Am J Kidney Dis* 39:274-282.

134. Pecoits-Filho, R., Barany, P., Lindholm, B., Heimburger, O., and Stenvinkel, P. 2002. Interleukin-6 is an independent predictor of mortality in patients starting dialysis treatment. *Nephrol Dial Transplant* 17:1684-1688.
135. Bologa, R.M., Levine, D.M., Parker, T.S., Cheigh, J.S., Serur, D., Stenzel, K.H., and Rubin, A.L. 1998. Interleukin-6 predicts hypoalbuminemia, hypocholesterolemia, and mortality in hemodialysis patients. *Am J Kidney Dis* 32:107-114.
136. Harris, T.B., Ferrucci, L., Tracy, R.P., Corti, M.C., Wacholder, S., Ettinger, W.H., Jr., Heimovitz, H., Cohen, H.J., and Wallace, R. 1999. Associations of elevated interleukin-6 and C-reactive protein levels with mortality in the elderly. *Am J Med* 106:506-512.
137. Nawawi, H., Osman, N.S., Annuar, R., Khalid, B.A., and Yusoff, K. 2003. Soluble intercellular adhesion molecule-1 and interleukin-6 levels reflect endothelial dysfunction in patients with primary hypercholesterolaemia treated with atorvastatin. *Atherosclerosis* 169:283-291.
138. Huber, S.A., Sakkinen, P., Conze, D., Hardin, N., and Tracy, R. 1999. Interleukin-6 exacerbates early atherosclerosis in mice. *Arterioscler Thromb Vasc Biol* 19:2364-2367.
139. Schieffer, B., Selle, T., Hilfiker, A., Hilfiker-Kleiner, D., Grote, K., Tietge, U.J., Trautwein, C., Luchtefeld, M., Schmittkamp, C., Heeneman, S., et al. 2004. Impact of interleukin-6 on plaque development and morphology in experimental atherosclerosis. *Circulation* 110:3493-3500.
140. Xing, Z., Gauldie, J., Cox, G., Baumann, H., Jordana, M., Lei, X.F., and Achong, M.K. 1998. IL-6 is an antiinflammatory cytokine required for controlling local or systemic acute inflammatory responses. *J Clin Invest* 101:311-320.
141. Aderka, D., Le, J.M., and Vilcek, J. 1989. IL-6 inhibits lipopolysaccharide-induced tumor necrosis factor production in cultured human monocytes, U937 cells, and in mice. *J Immunol* 143:3517-3523.
142. Bode, J.G., Nimmesgern, A., Schmitz, J., Schaper, F., Schmitt, M., Frisch, W., Haussinger, D., Heinrich, P.C., and Graeve, L. 1999. LPS and TNFalpha induce SOCS3 mRNA and inhibit IL-6-induced activation of STAT3 in macrophages. *FEBS Lett* 463:365-370.
143. Yuan, J., Wegenka, U.M., Lutticken, C., Buschmann, J., Decker, T., Schindler, C., Heinrich, P.C., and Horn, F. 1994. The signalling pathways of interleukin-6 and gamma interferon converge by the activation of different transcription factors which bind to common responsive DNA elements. *Mol Cell Biol* 14:1657-1668.
144. Stahl, N., Boulton, T.G., Farruggella, T., Ip, N.Y., Davis, S., Witthuhn, B.A., Quelle, F.W., Silvennoinen, O., Barbieri, G., Pellegrini, S., et al. 1994. Association and activation of Jak-Tyk kinases by CNTF-LIF-OSM-IL-6 beta receptor components. *Science* 263:92-95.
145. Guschin, D., Rogers, N., Briscoe, J., Witthuhn, B., Watling, D., Horn, F., Pellegrini, S., Yasukawa, K., Heinrich, P., Stark, G.R., et al. 1995. A major role for the protein tyrosine kinase JAK1 in the JAK/STAT signal transduction pathway in response to interleukin-6. *Embo J* 14:1421-1429.
146. Akira, S., Nishio, Y., Inoue, M., Wang, X.J., Wei, S., Matsusaka, T., Yoshida, K., Sudo, T., Naruto, M., and Kishimoto, T. 1994. Molecular cloning of APRF, a novel IFN-

stimulated gene factor 3 p91-related transcription factor involved in the gp130-mediated signaling pathway. *Cell* 77:63-71.
147. Heinrich, P.C., Behrmann, I., Haan, S., Hermanns, H.M., Muller-Newen, G., and Schaper, F. 2003. Principles of interleukin (IL)-6-type cytokine signalling and its regulation. *Biochem J* 374:1-20.
148. Levy, D.E., and Darnell, J.E., Jr. 2002. Stats: transcriptional control and biological impact. *Nat Rev Mol Cell Biol* 3:651-662.
149. Ihle, J.N. 2001. The Stat family in cytokine signaling. *Curr Opin Cell Biol* 13:211-217.
150. Stephanou, A., and Latchman, D.S. 2005. Opposing actions of STAT-1 and STAT-3. *Growth Factors* 23:177-182.
151. Darnell, J.E., Jr. 1997. STATs and gene regulation. *Science* 277:1630-1635.
152. Costa-Pereira, A.P., Tininini, S., Strobl, B., Alonzi, T., Schlaak, J.F., Is'harc, H., Gesualdo, I., Newman, S.J., Kerr, I.M., and Poli, V. 2002. Mutational switch of an IL-6 response to an interferon-gamma-like response. *Proc Natl Acad Sci U S A* 99:8043-8047.
153. Yasukawa, H., Ohishi, M., Mori, H., Murakami, M., Chinen, T., Aki, D., Hanada, T., Takeda, K., Akira, S., Hoshijima, M., et al. 2003. IL-6 induces an anti-inflammatory response in the absence of SOCS3 in macrophages. *Nat Immunol* 4:551-556.
154. Wormald, S., Zhang, J.G., Krebs, D.L., Mielke, L.A., Silver, J., Alexander, W.S., Speed, T.P., Nicola, N.A., and Hilton, D.J. 2006. The comparative roles of suppressor of cytokine signaling-1 and -3 in the inhibition and desensitization of cytokine signaling. *J Biol Chem* 281:11135-11143.
155. Sengupta, T.K., Talbot, E.S., Scherle, P.A., and Ivashkiv, L.B. 1998. Rapid inhibition of interleukin-6 signaling and Stat3 activation mediated by mitogen-activated protein kinases. *Proc Natl Acad Sci U S A* 95:11107-11112.
156. Gosling, J., Slaymaker, S., Gu, L., Tseng, S., Zlot, C.H., Young, S.G., Rollins, B.J., and Charo, I.F. 1999. MCP-1 deficiency reduces susceptibility to atherosclerosis in mice that overexpress human apolipoprotein B. *J Clin Invest* 103:773-778.
157. Ramana, C.V., Gil, M.P., Schreiber, R.D., and Stark, G.R. 2002. Stat1-dependent and -independent pathways in IFN-gamma-dependent signaling. *Trends Immunol* 23:96-101.
158. Hack, C.E., Aarden, L.A., and Thijs, L.G. 1997. Role of cytokines in sepsis. *Adv Immunol* 66:101-195.
159. Franscini, N., Bachli, E.B., Blau, N., Leikauf, M.S., Schaffner, A., and Schoedon, G. 2004. Gene expression profiling of inflamed human endothelial cells and influence of activated protein C. *Circulation* 110:2903-2909.
160. Hack, C.E., and Zeerleder, S. 2001. The endothelium in sepsis: source of and a target for inflammation. *Crit Care Med* 29:S21-27.
161. Yudkin, J.S., Kumari, M., Humphries, S.E., and Mohamed-Ali, V. 2000. Inflammation, obesity, stress and coronary heart disease: is interleukin-6 the link? *Atherosclerosis* 148:209-214.
162. Hansson, G.K. 2001. Immune mechanisms in atherosclerosis. *Arterioscler Thromb Vasc Biol* 21:1876-1890.
163. Williams, L., Jarai, G., Smith, A., and Finan, P. 2002. IL-10 expression profiling in human monocytes. *J Leukoc Biol* 72:800-809.

164. Guenzi, E., Topolt, K., Cornali, E., Lubeseder-Martellato, C., Jorg, A., Matzen, K., Zietz, C., Kremmer, E., Nappi, F., Schwemmle, M., et al. 2001. The helical domain of GBP-1 mediates the inhibition of endothelial cell proliferation by inflammatory cytokines. *EMBO J* 20:5568-5577.
165. Fulda, S., and Debatin, K.M. 2002. IFNgamma sensitizes for apoptosis by upregulating caspase-8 expression through the Stat1 pathway. *Oncogene* 21:2295-2308.
166. Widney, D.P., Xia, Y.R., Lusis, A.J., and Smith, J.B. 2000. The murine chemokine CXCL11 (IFN-inducible T cell alpha chemoattractant) is an IFN-gamma- and lipopolysaccharide-inducible glucocorticoid-attenuated response gene expressed in lung and other tissues during endotoxemia. *J Immunol* 164:6322-6331.
167. Neish, A.S., Read, M.A., Thanos, D., Pine, R., Maniatis, T., and Collins, T. 1995. Endothelial interferon regulatory factor 1 cooperates with NF-kappa B as a transcriptional activator of vascular cell adhesion molecule 1. *Mol Cell Biol* 15:2558-2569.
168. Marx, N., Mach, F., Sauty, A., Leung, J.H., Sarafi, M.N., Ransohoff, R.M., Libby, P., Plutzky, J., and Luster, A.D. 2000. Peroxisome proliferator-activated receptor-gamma activators inhibit IFN-gamma-induced expression of the T cell-active CXC chemokines IP-10, Mig, and I-TAC in human endothelial cells. *J Immunol* 164:6503-6508.
169. Chan, H., and Reed, J.C. 2005. TRAF-dependent association of protein kinase Tpl2/COT1 (MAP3K8) with CD40. *Biochem Biophys Res Commun* 328:198-205.
170. Warton, K., Foster, N.C., Gold, W.A., and Stanley, K.K. 2004. A novel gene family induced by acute inflammation in endothelial cells. *Gene* 342:85-95.
171. Chou, H.H., Yumoto, H., Davey, M., Takahashi, Y., Miyamoto, T., Gibson, F.C., 3rd, and Genco, C.A. 2005. Porphyromonas gingivalis fimbria-dependent activation of inflammatory genes in human aortic endothelial cells. *Infect Immun* 73:5367-5378.
172. Harrison, J.R., Kelly, P.L., and Pilbeam, C.C. 2000. Involvement of CCAAT enhancer binding protein transcription factors in the regulation of prostaglandin G/H synthase 2 expression by interleukin-1 in osteoblastic MC3T3-E1 cells. *J Bone Miner Res* 15:1138-1146.
173. Granger, R.L., Hughes, T.R., and Ramji, D.P. 2000. Stimulus- and cell-type-specific regulation of CCAAT-enhancer binding protein isoforms in glomerular mesangial cells by lipopolysaccharide and cytokines. *Biochim Biophys Acta* 1501:171-179.
174. Cantwell, C.A., Sterneck, E., and Johnson, P.F. 1998. Interleukin-6-specific activation of the C/EBPdelta gene in hepatocytes is mediated by Stat3 and Sp1. *Mol Cell Biol* 18:2108-2117.
175. Caivano, M., Gorgoni, B., Cohen, P., and Poli, V. 2001. The induction of cyclooxygenase-2 mRNA in macrophages is biphasic and requires both CCAAT enhancer-binding protein beta (C/EBP beta) and C/EBP delta transcription factors. *J Biol Chem* 276:48693-48701.
176. Zhao, B., Stavchansky, S.A., Bowden, R.A., and Bowman, P.D. 2003. Effect of interleukin-1beta and tumor necrosis factor-alpha on gene expression in human endothelial cells. *Am J Physiol Cell Physiol* 284:C1577-1583.
177. Mayer, H., Bilban, M., Kurtev, V., Gruber, F., Wagner, O., Binder, B.R., and de Martin, R. 2004. Deciphering regulatory patterns of inflammatory gene expression from

interleukin-1-stimulated human endothelial cells. *Arterioscler Thromb Vasc Biol* 24:1192-1198.

178. Wulczyn, F.G., Naumann, M., and Scheidereit, C. 1992. Candidate proto-oncogene bcl-3 encodes a subunit-specific inhibitor of transcription factor NF-kappa B. *Nature* 358:597-599.
179. Nishiyama, K., Takaji, K., Kataoka, K., Kurihara, Y., Yoshimura, M., Kato, A., Ogawa, H., and Kurihara, H. 2005. Id1 gene transfer confers angiogenic property on fully differentiated endothelial cells and contributes to therapeutic angiogenesis. *Circulation* 112:2840-2850.
180. Tang, J., Gordon, G.M., Nickoloff, B.J., and Foreman, K.E. 2002. The helix-loop-helix protein id-1 delays onset of replicative senescence in human endothelial cells. *Lab Invest* 82:1073-1079.
181. Hanada, T., and Yoshimura, A. 2002. Regulation of cytokine signaling and inflammation. *Cytokine Growth Factor Rev* 13:413-421.
182. Levy, D.E., and Lee, C.K. 2002. What does Stat3 do? *J Clin Invest* 109:1143-1148.
183. Brender, C., Nielsen, M., Kaltoft, K., Mikkelsen, G., Zhang, Q., Wasik, M., Billestrup, N., and Odum, N. 2001. STAT3-mediated constitutive expression of SOCS-3 in cutaneous T-cell lymphoma. *Blood* 97:1056-1062.
184. Ramana, C.V., Gil, M.P., Han, Y., Ransohoff, R.M., Schreiber, R.D., and Stark, G.R. 2001. Stat1-independent regulation of gene expression in response to IFN-gamma. *Proc Natl Acad Sci U S A* 98:6674-6679.
185. Obaya, A.J., Mateyak, M.K., and Sedivy, J.M. 1999. Mysterious liaisons: the relationship between c-Myc and the cell cycle. *Oncogene* 18:2934-2941.
186. Li, J., Yu, B., Song, L., Eschrich, S., and Haura, E.B. 2007. Effects of IFN-gamma and Stat1 on gene expression, growth, and survival in non-small cell lung cancer cells. *J Interferon Cytokine Res* 27:209-220.
187. Tilg, H., Dinarello, C.A., and Mier, J.W. 1997. IL-6 and APPs: anti-inflammatory and immunosuppressive mediators. *Immunol Today* 18:428-432.
188. Luchtefeld, M., Schunkert, H., Stoll, M., Selle, T., Lorier, R., Grote, K., Sagebiel, C., Jagavelu, K., Tietge, U.J., Assmus, U., et al. 2007. Signal transducer of inflammation gp130 modulates atherosclerosis in mice and man. *J Exp Med* 204:1935-1944.
189. Erren, M., Reinecke, H., Junker, R., Fobker, M., Schulte, H., Schurek, J.O., Kropf, J., Kerber, S., Breithardt, G., Assmann, G., et al. 1999. Systemic inflammatory parameters in patients with atherosclerosis of the coronary and peripheral arteries. *Arterioscler Thromb Vasc Biol* 19:2355-2363.
190. Ku, N.O., and Mortensen, R.F. 1993. The mouse C-reactive protein (CRP) gene is expressed in response to IL-1 but not IL-6. *Cytokine* 5:319-326.
191. Badolato, R., Wang, J.M., Murphy, W.J., Lloyd, A.R., Michiel, D.F., Bausserman, L.L., Kelvin, D.J., and Oppenheim, J.J. 1994. Serum amyloid A is a chemoattractant: induction of migration, adhesion, and tissue infiltration of monocytes and polymorphonuclear leukocytes. *J Exp Med* 180:203-209.
192. Gabay, C., and Kushner, I. 1999. Acute-phase proteins and other systemic responses to inflammation. *N Engl J Med* 340:448-454.
193. Heinrich, P.C., Behrmann, I., Muller-Newen, G., Schaper, F., and Graeve, L. 1998. Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. *Biochem J* 334 (Pt 2):297-314.

194. Gerhartz, C., Heesel, B., Sasse, J., Hemmann, U., Landgraf, C., Schneider-Mergener, J., Horn, F., Heinrich, P.C., and Graeve, L. 1996. Differential activation of acute phase response factor/STAT3 and STAT1 via the cytoplasmic domain of the interleukin 6 signal transducer gp130. I. Definition of a novel phosphotyrosine motif mediating STAT1 activation. *J Biol Chem* 271:12991-12998.
195. Lai, C.F., Ripperger, J., Wang, Y., Kim, H., Hawley, R.B., and Baumann, H. 1999. The STAT3-independent signaling pathway by glycoprotein 130 in hepatic cells. *J Biol Chem* 274:7793-7802.
196. Uhlar, C.M., and Whitehead, A.S. 1999. Serum amyloid A, the major vertebrate acute-phase reactant. *Eur J Biochem* 265:501-523.
197. Durbin, J.E., Hackenmiller, R., Simon, M.C., and Levy, D.E. 1996. Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. *Cell* 84:443-450.
198. Ram, P.A., Park, S.H., Choi, H.K., and Waxman, D.J. 1996. Growth hormone activation of Stat 1, Stat 3, and Stat 5 in rat liver. Differential kinetics of hormone desensitization and growth hormone stimulation of both tyrosine phosphorylation and serine/threonine phosphorylation. *J Biol Chem* 271:5929-5940.
199. Horvath, C.M., and Darnell, J.E. 1997. The state of the STATs: recent developments in the study of signal transduction to the nucleus. *Curr Opin Cell Biol* 9:233-239.
200. Williams, L., Bradley, L., Smith, A., and Foxwell, B. 2004. Signal transducer and activator of transcription 3 is the dominant mediator of the anti-inflammatory effects of IL-10 in human macrophages. *J Immunol* 172:567-576.
201. El Kasmi, K.C., Holst, J., Coffre, M., Mielke, L., de Pauw, A., Lhocine, N., Smith, A.M., Rutschman, R., Kaushal, D., Shen, Y., et al. 2006. General nature of the STAT3-activated anti-inflammatory response. *J Immunol* 177:7880-7888.
202. Sheikine, Y.A., and Hansson, G.K. 2006. Chemokines as potential therapeutic targets in atherosclerosis. *Curr Drug Targets* 7:13-27.

Samenvatting in het Nederlands voor de niet-ingewijde lezer

Inleiding

We spreken over chronisch nierfalen als de nieren onvoldoende in staat zijn om de afvalstoffen te verwijderen uit het bloed. Wanneer 90 tot 95 procent van de nier is uitgevallen, is een niervervangende behandeling zoals dialyse of niertransplantatie noodzakelijk. Chronisch nierfalen is er de oorzaak van dat stoffen die normaal gesproken uit het bloed verwijderd zouden moeten zijn, zich toch in het bloed blijven ophopen en zo zorgen voor secondaire problemen zoals hart- en vaatziektes. In feite zijn de hart- en vaatziektes en dan vooral atherosclerose de belangrijkste doodsoorzaak bij nierpatiënten.

Atherosclerose is een ziekte van de grote en middelgrote arteriën, waarbij sprake is van een ontstekingsproces wat gekarakteriseerd wordt door de lokale ophoping van vet, macrofagen en lymfocyten (die tot de circulerende witte bloedcellen behoren), bindweefsel en gladde spiercellen in de vaatwand. Deze ophoping in de vaatwand kan leiden tot een vernauwing van het betreffende vat met alle gevolgen van dien. Het atherosclerotisch proces, ook wel een atherosclerotische plaque genoemd, bevindt zich in de tunica intima van een arterie, die weer de binnenste laag van arterie vormt. Deze laag scheidt zicht van het bloed af door een laagje endotheelcellen en vormt een barrière die bijvoorbeeld ervoor zorgt dat monocyten en lymfocyten in het bloed blijven. Als endotheelcellen beschadigd zijn, verandert dat het karakter van deze cellen en verzwakt de eigenschap van de barrière. In dit geval trekken beschadigde endotheelcellen de monocyten en lymfocyten naar zich toe . Dit is de beginfase van het atherosclerotische proces.

Dat atherosclerose frequent in nierpatiënten voorkomt, heeft te maken met het feit dat nierpatiënten bepaalde risicofactoren hebben die de kans op atherosclerose vergroot zoals bloedarmoede, hoge bloeddruk en ontsteking. Recente studies tonen aan dat ontsteking, die een nierpatiënt oploopt, een belangrijke rol speelt in het ontstaan van atherosclerose. Verhoogde ontsteking of inflammatie in nierpatiënten kan worden aangetoond door het meten van het bloedgehalte van bepaalde ontstekingfactoren, die ook cytokinen worden genoemd zoals interleukine 6, en tumor necrosis factor alfa. Cytokinen kunnen het inflammatie proces versnellen of vertragen. Cytokinen die het inflammatie proces

versnellen worden pro-inflammatoir genoemd (zoals tumor necrosis factor alfa en interferon gamma) en cytokinen die het inflammatie proces vertragen worden anti-inflammatoire cytokinen genoemd (zoals interleukine 10). In het algemeen geldt dat als het niveau van pro-inflammatoire cytokinen verhoogd is, de anti-inflammatoire cytokinen worden gemaakt die de balans tussen pro- en anti-inflammatoire cytokinen herstellen. Echter, als de balans tussen deze twee soorten cytokinen verstoord blijft, kan dit tot inflammatoire ziektes zoals reumatoïde artritis en atherosclerose leiden. Omdat inflammatie in nierpatiënten verhoogd is, geeft dit aan dat de productie van pro-inflammatoire cytokinen hoger is dan die van anti-inflammatoire cytokinen. Dit kan een belangrijke rol spelen in verhoogde atherosclerose in nierpatiënten. In dit geval is het belangrijk om naar andere mogelijkheden te zoeken die de balans tussen pro- en anti-inflammatoire cytokines kunnen herstellen en het inflammatie proces in nierpatiënten zouden kunnen vertragen. Om dit te realiseren, zouden we dan eerst moeten weten hoe cytokinen werken.

Op het celoppervlak zijn verschillende cytokine receptoren aanwezig. Iedere cytokine heeft zo zijn eigen receptor. Nadat een cytokine een verbinding heeft gemaakt met zijn specifieke receptor, leidt dit tot intracellulaire activatie van een aantal eiwitten, waaronder transcriptirfactoren van de STAT familie (wat voor “Signal transducer and activator of transcription” staat). Deze moleculen kunnen zich vervolgens verplaatsen naar de kern, om daar te binden aan DNA en te zorgen dat van bepaalde genen mRNA kopieën worden gemaakt. Later wordt in het cytoplasma op basis van deze gekopieerde genen nieuwe eiwitten gemaakt, die bepaalde functies van de cel (zoals cytokinen productie en migratie) kunnen beïnvloeden. De activiteit van STAT transcriptiefactoren leidt ook tot de inductie van een speciale familie van eiwitten die SOCS worden genoemd (SOCS is de afkorting voor “suppressors of cytokine signaling”). SOCS eiwitten zijn in staat de werking van STAT transcriptiefactoren te remmen en vormen zo eigenlijk de natuurlijke procesremmers van cytokinen. Omdat de productie van SOCS eiwitten het gevolg is van STAT activiteit (die door cytokinen geactiveerd is), kan verhoogde SOCS in de cel aangeven dat deze cel onder invloed van cytokine(n) geweest is. Tot dusver zijn er 8 eiwitten bekend die tot de SOCS familie behoren namelijk SOCS1 t/m 7 en CIS. Het is belangrijk ons te realiseren dat normaliter SOCS eiwitten de werking van zowel van pro- als anti-inflammatoire cytokinen kunnen remmen. Als de werking van anti-inflammatoire cytokinen geremd wordt, of als de werking van pro-

inflammatoire cytokinen onvoldoende geremd wordt, leidt dit tot verhoogde inflammatie. In nierpatiënten bestaan aanwijzingen dat de werking van belangrijke anti-inflammatoire factoren verminderd is. De oorzaak hiervan is echter niet duidelijk, maar zou mogelijk verband met de aanwezigheid van te veel SOCS eiwitten in bepaalde cellen. Daarom denken we dat verhoogde SOCS in bepaalde celtypen zou betrokken zijn bij verhoogde inflammatie in nierpatiënten en vervolgens in de ontwikkeling van atherosclerose.

Het proefschrift

Dit proefschrift bestudeert de relatie tussen SOCS eiwitten en inflammatie in nierpatiënten en eveneens hoe verhoogde SOCS eiwitten de werking van cytokinen kan beïnvloeden.

Hoofdstuk 1 geeft een uitgebreide inleiding over chronisch nierfalen en de daarbij behorende gevolgen zoals verhoogde atherosclerose. Verder is er beschreven hoe verhoogde inflammatie de functie van zowel bloedcellen als endotheelcellen in een pro-inflammatoire richting verandert. Verder wordt ook veel aandacht besteedt aan de invloed van cytokinen op de functie van hun target cellen door het activeren van STAT eiwitten en inductie van SOCS eiwitten. In **hoofdstuk 1** wordt verder beschreven dat veranderingen in SOCS expressie aan de basis van een aantal inflammatoire ziektes liggen. Ook wordt de vraag gesteld of SOCS betrokken kan zijn bij de ontwikkeling van hart- en vaatziektes. Ten slotte worden de algemene hypothese en vragen van deze studie beschreven.

Eerst is de hypothese getest of verhoogde inflammatie in CKD patiënten gepaard gaat met verhoogde SOCS in monocyten en lymfocyten. (CKD, wat voor “chronic kidney disease” staat verwijst naar patiënten die nog geen dialyse behandeling hebben gehad). Omdat deze cellen in direct contact zijn met verhoogde systemische inflammatie **(hoofdstuk 2)**. Inderdaad hebben we aangetoond dat in CKD patiënten SOCS3 verhoogd is in monocyten en SOCS1 in lymfocyten. Tevens hebben we aangetoond dat verhoogde SOCS1 in lymfocyten correleert met verhoogde tumor necrosis factor alfa (een pro-inflammatoire cytokine) in het bloed. Dit bewijst dat SOCS verhoogd is in circulerende witte bloedcellen van CKD patienten en dat dit mogelijk het gevolg is van verhoogde inflammatie. Vervolgens hebben we met behulp van statistische testen gekeken of er correlaties bestonden tussen verhoogde SOCS in

circulerende bloedcellen en risicofactoren van hart- en vaatziektes zoals bloeddruk, verminderde nierfunctie en de flexibiliteit van de vaatwanden. Deze analyses toonden aan dat verhoogde SOCS3 in monocyten correleert met verminderde nierfunctie, terwijl verhoogde SOCS1 in lymfocyten correleert met verhoogde bloeddruk, in flammatie en verminderde flexibiliteit van de vaatwanden. Omdat verminderde nierfunctie, verhoogde bloeddruk, verhoogde inflammatie en verminderde flexibiliteit van de vaatwanden bekend zijn als risicofactoren van hart- en vaatziektes, stellen we voor dat verhoogde SOCS een nieuwe intracellulaire marker voor hart- en vaatziektes in nierpatiënten zou kunnen zijn.

Vervolgens wordt dezelfde hypothese dat verhoogde inflammatie gepaard gaat met verhoogde SOCS in circulerende witte bloedcellen getest in ESRD (wat voor “end stage renal disease” staat) patiënten die in eindstadium van nierfalen verkeren **(hoofdstuk 3)**. Bij deze patiënten is de functie van de nieren zodanig verminderd dat dialyse onvermijdelijk is. Verder is de inflammatie bij deze patiënten nog hoger dan CKD patiënten, wat te maken zou kunnen hebben met het dialyse proces. We vonden zowel verhoogde SOCS1 in monocyten als lymfocyten en verhoogde CIS-1 in lymfocyten van ESRD patiënten. Verder constateerden we dat verhoogde SOCS1 in monocyten met verhoogde interleukine 6 (een cytokine die beide pro- en anti-inflammatoire effecten heeft) correleert. Dit bevestigt dat verhoogde inflammatie in nierpatiënten gepaard gaat met verhoogde SOCS in circulerende cellen.

Hoewel deze data onze hypothese bevestigen, leggen ze geen direct verband tussen verhoogde SOCS in monocyten en lymfocyten en het ontstaan van atherosclerose in nierpatiënten. Desondanks zijn er studies met muizen uitgevoerd, die de aanwezigheid van SOCS1 en SOCS3 in atherosclese plaques hebben aangetoond. Bovendien toont één van deze studies aan dat de afwezigheid van SOCS3 in monocyten in het atherosclerose muizen model gepaard gaat met verminderde atherosclerose. Deze studie legt een direct verband tussen SOCS expressie in monocyten en het atherosclerose proces en introduceert SOCS3 in monocyten als een pro-atherosclerose factor. In onze studie hebben we aangetoond dat SOCS3 in monocyten van CKD patiënten verhoogd is en niet bij ESRD patiënten. En dus is het interessant om verder uit te zoeken of verhoogde SOCS3 in monocyten van CKD patiënten gepaard gaat met verhoogde atherosclerose. In tegenstelling tot SOCS3 kan SOCS1 het ontstaan van atherosclerose vertragen en dat heeft te maken met het remmende effect van SOCS1 op een aantal pro-inflammatoire cytokinen (o.a. interferon gamma). We vermelden

hier de verhoogde SOCS1 in monocyten van ESRD patiënten. Desondanks kan onze data niet aangeven of verhoogde SOCS1 in monocyten van ESRD patiënten inderdaad gepaard gaat met verminderde atherosclerose. Dit zou dan ook in een vervolg studie moeten worden onderzocht.

In de **hoofdstukken 4 en 5** beschrijven we hoe verhoogde SOCS in een bepaalde cellijn de response van de cel op andere cytokinen beïnvloedt. Zoals hierboven besproken is onder inflammatoire omstandigheden (zoals nierfalen) het bloedgehalte van bepaalde inflammatoire cytokinen verhoogd. Gevolg van dit fenomeen is dat cellen die direct contact hebben met het bloed zoals bloedcellen en cellen die de vaatwand bedekken (endotheelcellen) bloot worden gesteld aan een aantal cytokinen. Deze cytokinen kunnen elkaars werking versterken of verzwakken. Als bijvoorbeeld twee cytokinen via dezelfde STAT(s) werken, kan hun gelijktijdige signaal verstrekt worden. SOCS eiwitten die echter door een cytokine worden opgereguleerd, kunnen zo de werking van een andere cytokine(n) verminderen. In dit verband hebben we in een humane endotheelcellijn de interacties tussen twee cytokinen bestudeerd die een belangrijke rol bij de ontwikkeling van atherosclerose spelen, namelijk: interleukine 6 en interferon gamma. In eerste instantie hebben we onderzocht welke STAT door interferon gamma of interleukine 6 in endotheelcellen wordt geactiveerd en welke SOCS door ieder cytokine worden geinduceerd. Vervolgens hebben we de interacties tussen deze twee cytokinen onderzocht **(hoofdstuk 4)**. Onze data toonde aan dat stimulatie van endotheelcellen door interferon gamma tot activiteit van STAT3, en voornamelijk STAT1 leidt, en dat stimulatie door interleukine 6 tot activiteit van STAT3 leidt. Verder is aangetoond dat interferon gamma stimulatie van endotheelcellen tot inductie van SOCS3 en voornamelijk SOCS1 leidt, terwijl interleukine 6 stimulatie tot inductie van SOCS3 leidt. SOCS1 remt voornamelijk de activiteit van STAT1 en SOCS3 remt de activiteit van STAT3. Vervolgens werden endotheelcellen eerst behandeld met interferon gamma en vervolgens met interleukine 6. De belangrijkste bevinding was dat de voorbehandeling van endotheelcellen met interferon gamma, de activiteit van STAT3 door interleukine 6 vermindert. In dit verband hebben we met behulp van verschillende technieken aangetoond dat dit remmende effect afhankelijk is van SOCS3 inductie door interferon gamma. Omdat voornamelijk STAT3 activiteit verantwoordelijk is voor het induceren van target genen van IL-6, hebben we uitgezocht hoe deze verminderde STAT3 activiteit de expressie van interleukine

6 target genen (die pro- of anti- inflammatoire effecten kunnen hebben) beïnvloedt **(hoofdstuk 5)**. Onze data suggereert dat de communicatie over en weer tussen interferon gamma en interleukine 6 (die SOCS3 afhankelijk is) ervoor zorgt dat de expressie van STAT3 target genen - die beschermende effecten voor endotheelcel zouden kunnen hebben verminderd is, terwijl de expressie van bepaalde genen (die waarschijnlijk STAT3 onafhankelijk zijn) die pro-inflammatoire invloeden zouden kunnen hebben ongedeerd bleven of zelfs verhoogd waren. Dit zou een mechanisme kunnen zijn waarbij het functioneren van endotheelcellen wordt verstoord en dit zou weer kunnen bijdragen aan de ontwikkeling van atherosclerose.

Het is ook belangrijk te weten dat activiteit van STAT1 en STAT3 tot tegengestelde effecten leidt. In de meeste gevallen leidt STAT1 activiteit tot celdood en pro-inflammatoire effecten, terwijl STAT3 activiteit de levensduur van de cellen verlengt en het zou ook andere anti-inflammatoire effecten kunnen hebben. Eerdere studies hebben aangetoond dat in afwezigheid van STAT3, interleukine 6 via STAT1 kan werken, en in dit geval interleukine 6 meer proinflammatoire effecten heeft. Omdat interleukine 6 zowel pro- als anti-inflammatoire effecten heeft, hebben we in **hoofdstuk 6** de hypothese getest dat STAT1 een belangrijke rol in de werking van interleukine 6 speelt en dat STAT1 verantwoordelijk zou kunnen zijn voor pro-inflammatoire effecten van interleukine 6. Om deze hypothese te testen hebben we gebruik gemaakt van bepaalde muizen modellen die geen STAT1 bevatten. Onze voorlopige data in **hoofdstuk 6** toont aan dat de expressie van bepaalde target genen (zoals SAA1; een eiwit die betrokken is bij inflammatie proces) van interleukine 6 behoorlijk wordt verlaagd in afwezigheid van STAT1, wat weer aantoont dat STAT1 een belangrijke rol in de werking en effecten van interleukine 6 speelt. Deze studie zou echter met een groter aantal muizen moeten worden herhaald.

In **hoofdstuk 7** zijn de bevindingen van de gehele studie op een rijtje gezet en is uitgelegd wat die voor nierpatiënten zou kunnen betekenen en waarop eventuele vervolg studies zich moeten richten om de rol van SOCS als een marker van hart- en vaatziektes in nierpatiënten en de rol van SOCS als een therapeutische mogelijkheid voor atherosclerose te kunnen bevestigen.

Acknowledgments

My dear family;

Many years have been passed since I've left our home to study Pharmacy at Ahwaz University, the day that I left our home to discover myself and to be the man that I have become. Through all these years you have supported me regardless what I was doing. There was always concern about me; does he eat well? Does he have enough money? …I felt always your love, friendship, and compassion. My dear parents thank you for all you have done for me. Dear **Mohsen**, I believe the choice that you have made in your life, affected us all. Thank you for being such a good example. My dear **Zahra, Giti** (who could believe that you will get your Ph.D!?), **Hadi, Azar, Farzaneh, Sima, Masoud, Marjan, and Mahmoud**, how could I imagine having a happy life without you? And how could I have achieved anything without your support? Thank you for everything.

Dear **Prof. dr. Moore,** I should express my appreciation for your friendship and concern for me and my family.

Mijn beste promotor, co-promotors en stafleden van Nefrologie, vasculaire geneeskunde en Experimentele cardiologie van het UMC Utrecht

Beste **Prof. Pasterkamp**. Het is mij een eer dat u ondanks uw drukke agenda mij als promotor bij hebt gestaan.

Beste **Hans en Branko**: Ten eerste moet ik jullie zeer bedanken voor jullie vertrouwen in mij. In 2004 toen ik hoorde dat ik als AIO bij jullie mocht komen werken, werd ik overvallen door twijfels. Ik had toen een fatsoenlijk en goed betaalde baan. Jullie hebben mij toch overtuigd dat ik deze uitdaging (AIOschap) aan moest gaan. Hans zei: "je wordt met een Ph.D in je zak alleen maar beter". Ik begon met dit project met die gedachten dat ik "er beter van zou worden". Er zijn vele momenten geweest dat ik dacht "waarmee ben ik in God's naam begonnen" maar ik heb nooit gedacht dat ik beter zou moeten stoppen. Nu, vier jaar later ben ik blij dat dit project netjes afgerond is. Maar dat is niet alleen mijn "achievement" maar ook die van jullie. Het was niet makkelijk om mij van afstand te begeleiden. Maar misschien was dit toch de beste optie. Ik ben natuurlijk behoorlijk koppig en eigenwijs, ondanks dit wisten jullie mij toch op de goede weg te houden en helpen. Branko! jou reactie op de eerste artikel dat ik geschreven had was dit: " Dit is geen Engels!", en inderdaad het was geen goede Engels. Desondanks, mede te danken aan jullie inzet, ligt hier nu een proefschrift waarop ik trots mag zijn (en hopelijk jullie ook). Dank voor jullie support, geduld, en vooral voor wat jullie mij bij hebben gebracht.

Beste **Walther en Peter Boer, Jaap, Peter Blankestijn, Marianne en Marie-Jose:**
Jullie hulp in de afgelopen vier jaar was onmisbaar. Mijn gesprekken met **Walther en Peter** waren altijd kort, maar ik kreeg altijd mijn antwoord en hulp die ik nodig had. super!. Beste **Jaap**! Ik was altijd verbaasd hoe snel je reageerde op artikelen die je te zien kreeg. Dank voor je inzet en onmisbare bijdrage!, Beste **Peter Blankestijn**; het was niet mogelijk om de dialyse patiënten te rekruteren als je ermee niet geholpen had, dank je wel. Beste **Marianne en Marie-Jose**; dank voor alle gesprekken en goede tips die jullie me hebben gegeven.

My Dear Friends in Iran;

Having a good friend is a gift, I was so lucky to have a few good friends. Dear **Alireza Razavi**! do you remember the night that we were walking on the Ordibehesht street talking about the ever existing dilemma "money or science"? We were 14 years old. You said that you wanted to be a businessman with your own company, a rich man. I said that "I want to be a doctor; it will make me rich anyway!". Now you have your own company, and I received for the second time the title of "doctor"; once in Iran and now in Holland. However, I am not rich at all! But I am glad to see that we were able to realize our dreams. Dear **Ahmad Rasouli**; the mustache-guy among us, just your mustache made our circle at school much stronger than the others! I will never forget what you have done during the first days of our military service; switching between the groups to be in my group. Although you knew that it was the group that was supposed to be trained much tougher; in order to do difficult tasks. You did the training as good as you could, and I escaped of doing it as much as I could! I was not surprised years later when I heard you were doing your Ph.D in immunology at Shiraz University. Dear **Gholamreza zare**; the most gentle-guy among us. We experienced a lot of adventures together; I have still many pictures of our adventures in the nature. I am happy to see that you have become what you wanted to be; a successful lawyer. Dear **Mohsen Amiri**! Do you remember the theater that we made at Ahwaz University? It was such a nice experience. Now that I am trying to translate your play "Adam and Eva" to Dutch language, I can not resist the temptation to imagine how it could be to perform your play in Holland. Dear **Alireza-Ghasemi**! I met you when I was a second year pharmacy student, and you were a co-assistant. Our friendship grew so fast and you became one of my best friends ever and that means a lot to me. During the first difficult years in Holland I was always more than happy to receive a letter from you. Dear **Bahare!** It was such a nice experience to receive your packages full of dried Persian vegetables, and candies in Holland. Thank you.

Mijn beste vrienden in Holland;

Beste **Ewout and Ingrid**; mijn leven in Nederland zou ontzettend moeilijker zijn geweest als ik niet in de eerste maanden van mijn verblijf in Nederland jullie had leren kennen. De eerste kerst dagen in Nederland mocht ik met jullie en jullie familie vieren. Vele oud-nieuw avonden hebben we samen gevierd. Ik heb nog steeds dat notitieboek, waarin ik elke week Nederlandse woorden opschreef die ik niet begreep om die vervolgens met jullie te bespreken. Dank is een zeer klein woord voor alles wat jullie tot nu toe voor mij gedaan hebben, maar toch mijn woordenschat beperkt zich tot " dank en dank en nogmaals dank".

Tanja, Monica, Julia, Mascha en Audrey; ik zal me niet vergeven als ik jullie hier niet bedankt voor zoveel momenten van geluk die jullie me geschonken hebben. Jullie zullen voor altijd in mijn gedachten blijven.

Beste **Farid en Nina**; mijn Iraanse vrienden in Holland, jammer dat jullie naar Iran zijn verhuisd. Beste Farid, ik heb altijd een grote bewondering voor alles wat je in wetenschap en ook in business bereikt hebt. Als ik ooit iets in de business zou bereiken, zou ik dat aan je te danken hebben. Beste Nina, je bent een dierbare vriendin en een heel goede kok!! Dank voor alle lekkere Perzische gerechten!

Beste **Richard Huibregts en Suzanne**: Leuk dat jullie uiteindelijk een huis gekocht hebben, gefeliciteerd. **Richard!** door die jaren van de AIO-schap hebben we elkaar beter leren kennen. Je bent een goede voetballer, een goede arts, een goede wetenschapper en een zeer dierbare vriend. Het was leuk om met je over alles en nog wat te kletsen. Zeker als we even

koffie hadden gehaald bij de koffiemachine, hadden we altijd iemand gezien die een geklets waard was!

Beste **Lars van Oerthel**; mijn huisgenoot op Rubenslaan en mijn gitaarleraar! Ik Ben ontzettend blij dat ik een tijdje in die flat op Rubenslaan mocht wonen en je leren kennen. Het is zeer leuk om samen met je naar een film te kijken, borrelen, en zeker over dames te kletsen!

Beste collega's van Nefrologie en vasculaire geneeskunde

Beste **Nel, Adéle, Chantal, Marloes en Petra**: Fantastische analisten van onze lab. Bedankt voor al jullie inzet, medewerking, samenwerking en gezelligheid. Het was onmogelijk om al die patiënten materialen op te slaan en te verwerken als Nel niet erbij was. Het was onmogelijk om al die experimenten met IFNγ en IL-6 uit te voeren als Chantal niet erbij was. Ik ben ontzettend dankbaar voor jullie inzet. Adéle, je hebt zoveel experimenten uitgevoerd met IFNγ en H_2O_2, jammer dat die data niet terecht gekomen zijn in dit proefschrift, maar ik ben je ontzettend dankbaar voor alle klussen die je voor me hebt gedaan. Beste **Coby**, dank voor al je hulp voor het meten van PWV en bloedafname. Beste **Anne**, het was mijn genoegen om af en toe in de gang in het Farsi met je te mogen kletsen. Ik ben ontzettend blij dat ik je bij Nefro ontmoet heb.

Laima, Sebastian, Walter, Arijan, Olivia, Kim, Ralf, Peter, Helena, Neelke, Lenart, Maarten, Arianne, Albert, Lars, Bas, en Joost: Ik ben ontzettend blij dat ik veel leuke mensen in het UMC utrecht ontmoet heb. Het is leuk om een paar AIO's om me heen te hebben om over alles en nog wat te klagen. Ik ben jullie dankbaar voor al jullie hulp en medewerking….**Laima**! verwarming altijd op 30 graden!! Ben inmiddels een beetje gekookt! Ik heb ontzettend respect voor de persoon die je bent. **Sebastian**! Sociaal en (heel soms) grappig en ontzettend aardige collega. Het verhaal van "ouwehoeren" zal ik nooit vergeten. **Walter**! Dank voor je CD! **Arijan**: succes met het afronden van je promotie. **Olivia**! Het was altijd leuk om je even gesproken te hebben. Ben benieuwd wat je straks na je promotie gaat doen. **Kim**: altijd vrolijk en lachend! **Helena**: Ben blij dat je de baan kreeg die je heel graag wou. **Neelke**: je was alleen maar een dag per week of soms per weken bij ons. Maar ik was altijd blij om je weer te zien. We spreken elkaar zeker weer. **Lenart**: combinatie van vechtsport en wetenschap! **Maarten**! Ik heb altijd bewondering gehad voor je lange fietstochten. Hoe was het in Canada? **Arianne**: Net begonnen, maar altijd heel erg netjes en mooi aangekleed! **Lars:** succes met je avontuur in New York! **Bas en Joost:** succes met het opzetten van jullie projecten.

My dear colleagues and friends at NYU and in Central park west

Dear **Prof. Levy**: I am so grateful that I was allowed to work at your lab for a few months. It was a very special experience. Thank you very much.

Dear **Yaming, Blue, Valentina, and Dan**: **Yaming and Dan**: Thank you for all your help and friendship. Dear **Blue and Valentina**: I experienced NY as a wonderful city. It was a wonderful experience because I have had your friendship there. I was so lucky that Blue (and **Asya and Elisa**) lived just a few blocks away from my place. I have seen many movies, opera, dance performance, museums together with you. I am very grateful that I have met you there. I will certainly come back to New York just to meet you again.

Curriculum Vitae

Mehdi Rastmanesh was born in Shiraz an ancient city in southwest of Iran, where he obtained his primary and secondary school certificates. In 1991 he joined the army to fulfill his military services for two years. In 1993 he passed the National Admission Exam For Higher Education with excellent results and entered the University Of Medical Science Of Ahwas to study Pharmacy. He graduated from the School of Pharmacy of Ahwas University in February 1999 as a top student. He then worked as a pharmacist and Chief Medical Care Officer at the local office of the Ministry of Public Health of Iran. In November 2002 he proceeded to study Bio-Pharmaceutical Sciences at the University of Leiden, The Netherlands, where he obtained his Master degree in June 2003. He worked then as Registration Coordinator at the Dutch Medicine Evaluation Board. In September 2004 he accepted for PhD studies at the Department of Nephrology and Hypertension at Utrecht University, under supervision of Prof. dr. G. Pasterkamp, Dr. Branko Braam, en Dr. Hans Bluyssen. In November 2007, supported by a grant from Dutch Kidney Foundation he moved to New York to perform a part of his PhD research at New York University, Department of Pathology, Prof. Levy's Lab for four months.
Mehdi Rastmanesh Started his own company in Life Science (Sinagene) in July 2008 in The Netherlands.