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Regulation of Cytokine Signaling During Macrophage Differentiation and Activation

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ACADEMIC DISSERTATION

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1. ABSTRACT

Hematopoietic cytokines regulate the differentiation and growth of blood progenitor cells in bone marrow. As hydrophilic substances, cytokines cannot penetrate the cell membrane, but they use specific transmembrane receptors to transmit information from extracellular space into cells. The binding of a given cytokine into a corresponding transmembrane receptor triggers the specific activation of several intracellular signaling pathways.

Macrophages have an important role in regulating immune responses through phagocytosis and by expressing both pro- and anti-inflammatory cytokines. Macrophage development from an undifferentiated precursor cell into a fully active effector cell of innate immunity requires several steps of differentiation, activation and proliferation. Most of these cellular functions are tightly regulated by hematopoietic cytokines. This study was aimed at characterizing the changes in cytokine signaling - especially the negative regulation of signaling - during macrophage differentiation and activation.

An important cytokine for the proliferation of early myeloid precursor cells is interleukin-3 (IL-3). Binding of IL-3 to its cognate receptor on the cell surface leads to the activation of Janus kinase 2 (named after Janus, the ancient Roman god of gates and doorways who was termed Janus; hence Jak) and the subsequent activation of the Signal Transducer and Activator of Transcription 5 (Stat5) transcription factor. IL-3-induced Jak2/Stat5 activation promotes cell proliferation. During macrophage differentiation the IL-3-induced tyrosine phosphorylation of several intracellular proteins including both IL-3 receptor β-chain (IL-3Rβ) and Jak2 was inhibited in murine myeloid progenitor 32D cells. Differentiation in these cells was induced by the activation of ectopically expressed Protein kinase C (PKC)-α and -δ isoforms. PKC-δ is a serine/threonine kinase, whose kinase activity was required for Jak2 inhibition. It was observed that PKC-δ induced both serine and threonine phosphorylation of Jak2.

A key cytokine regulating macrophage activity is Interferon (IFN)-γ. IFN-γ is an important activator of monocytes and macrophages and it also regulates immunoglobulin class switch in B-cells and Th_1 differentiation from naïve Th_0 cells. IFN-γ receptor activation induces the activation of Jak1/Jak2 and Stat1. To study whether PKC-δ-mediated Jak2 inhibition was utilized during macrophage activation, IFN-γ-induced signaling was investigated. IFN-γ-induced Jak2 activation was inhibited by PKC-α and -δ isoforms in human promyelocytic HL-60 and THP-1 cells. In addition, human monocytes demonstrated decreased IFN-γ responsiveness upon PKC activation. As in the case of the IL-3 receptor, inhibition of IFN-γ signaling required the kinase activity of PKC-δ.

To study the role of PKC-δ in physiological signaling cascades leading to macrophage differentiation, the signaling of Macrophage-Colony Stimulating Factor (M-CSF), a cytokine that induces macrophage differentiation, was studied. M-CSF rapidly increased PKC-δ activity. In addition, Fcγ-receptor (FcγR), an activating macrophage receptor induced PKC-δ activity. M-CSF-induced differentiation corresponded to the rapid activation of PKC-δ, resulting in the increased expression of Protein kinase A related kinase (Pkare), a murine homologue of the novel human kinase, Protein Kinase X (PRKX), whose expression is associated with macrophage differentiation.

An important mechanism for the negative regulation of cytokine signaling is polyubiquitination. Protein degradation mediated by polyubiquitination is characterized by the ligation of ubiquitin proteins into target proteins which results in the degradation of the targets in 26 proteasomes, large multicatalytic proteases. An additional mechanism for down-regulating cytokine-induced Jak2 responses was found to be the ubiquitination and proteosomal degradation of tyrosine phosphorylated Jak2. In addition to the tyrosine phosphorylation of Jak2, ubiquitination involved suppressor of cytokine signaling1 (SOCS1).

Both ubiquitination and PKC-δ-mediated inhibition of Jak2 appear to represent mechanisms of the fine-tuning of cytokine signaling.

1. TIIVISTELMÄ

Hematopoieettiset sytokiinit säätelevät luuytimen kantasolujen kasvua ja erilaistumista. Sytokiinit ovat vesiliukoisia yhdisteitä eivätkä täten voi kulkea rasvoista koostuvan solukalvon läpi, vaan ne käyttävät erityisiä solukalvon läpäiseviä reseptoreita välittääkseen tietoa solunulkoisesta tilasta solun sisään. Sytokiinin sitoutuminen solukalvon läpäisevään reseptoriin aiheuttaa tiettyjen solunsisäisten tiedonvälitysreittien aktivoitumista.

Makrofageilla on tärkeä rooli immuunivasteen säätelyssä. Makrofagien pääasialliset tehtävät ovat fagosytoosi ja proinflammatoristen sytokiinien erittäminen. Makrofagien kehittyminen epäkypsästä luuytimen kantasolusta aktivoiduksi kudosmakrofagiksi vaatii useiden kasvu-, aktivoitumis- ja erilaistumismisvaiheiden läpikäyntiä. Useimpia näistä vaiheista säätelevät tarkasti hematopoieettiset sytokiinit. Tämän tutkimuksen tavoitteena on ollut selvittää erityisesti negatiivisen sytokiinisignaloinnin mekanismeja makrofagien erilaistumisen ja aktivoitumisen aikana.

Varhaisten myeloisten kantasolujen tärkeä kasvutekijä on interleukiini-3 (IL-3). IL-3:n sitoutuminen omaan reseptoriinsa solun pinnalla laukaisee solunsisäisen Jak2-kinaasin aktivaation, mikä puolestaan johtaa Stat5 transkriptiotekijän aktivoitumiseen. IL-3:n aiheuttama Jak2/Stat5-aktivaatio lisää solujen kasvua. Makrofagierilaistumisen aikana IL-3:n aiheuttama proteiinien tyrosiinifosforylaatio estyi hiiren myeloisissa 32D soluissa. Näihin proteiineihin kuuluivat mm. IL-3 reseptorin β-ketju ja Jak2-kinaasi. Erilaistumisen aiheutti näissä soluissa ylimääräisenä ilmennetyn Proteiini kinaasi C (PKC)-α tai -δ isoformin aktivaatio, jotka molemmat estivät IL-3 välittämän Jak2 aktivaation. PKC on seriini/treoniinikinaasiperhe, jonka jäsenen, PKC-δ:n, kinaasiaktiivisuutta vaadittiin Jak2:n toiminnalliseen estoon. Lisäksi PKC-δ aiheutti Jak2:n seriini- ja treoniiniaminohappotähteiden fosforylaatiota.

Interferoni-γ (IFN-γ) on tärkeä makrofagien aktiivisuuden säätelijä. IFN-γ aiheuttaa myös immunoglobuliinivasteen kyspsymistä B-soluissa, sekä Th1 tyyppisten T-solujen erilaistumista. IFN-γ reseptorin aktivaatio aiheuttaa Jak1/Jak2- ja Stat1-signalointireitin aktivoitumisen. Jotta tiedettäisiin, toimiiko PKC-δ-välitteinen Jak2-kinaasin negatiivinen säätelymekanismi makrofagien aktivoitumisen aikana, tutkittiin IFN-γ reseptorin signalointireittiä. IFN-γ:n aiheuttama Jak2-aktivaatio estyi PKC-α:n ja PKC-δ:n vaikutuksesta myeloisten solujen esiasteissa (HL-60 ja THP-1 soluissa). Lisäksi havaittiin ihmisen verestä eristettyjen monosyyttien vasteiden IFN-γ:lle heikkenevän jos PKC oli aktivoitu. Kuten IL-3:n kohdalla, IFN-γ signaloinnin esto vaati PKC-δ:n kinaasiaktiivisuutta.

PKC-δ:n merkitystä fysiologisissa signalointireiteissä jotka johtavat makrofagien erilaistumiseen tutkittiin seuraavaksi Macrophage Colony Stimulating Factor (M-CSF)-sytokiinin signaloinnissa. M-CSF säätelee makrofagien erilaistumista. PKC-δ aktivoitui nopeasti M-CSF:n vaikutuksesta. Lisäksi Fcγ reseptori (FcγR), makrofagien pintareseptori joka aktivoi makrofagien toimintaa, aktivoi myös PKC-δ:n. M-CSF:n välittämä erilaistuminen aktivoi PKC-δ:n nopeasti, ja lisäsi Protein kinase A related kinase (Pkare)-geenin luentaa. Pkare on hiiren homologi uudelle ihmisen kinaasille Protein Kinase X:lle (PRKX), jonka on havaittu olevan tärkeässä osassa makrofagien erilaistumisessa.

Tärkeä mekanismi sytokiinisignaloinnin negatiivisessa säätelyssä on polyubiquitinisaatio. Polyubiquitinaatio välitteisessä proteiinien hajottamisessa kohdeproteiiniin liitetään useita ubiquitin-proteiineja, jotka ohjaavat kohdeproteiiniin 26S proteasomille. 26S proteasomi on iso, katalyyttinen proteaasi, joka pilkkoo siihen ohjautuva proteiinit. Ubiquitinisaation ja proteosomaalinen tyrosiinifosforyloidun proteiinin hajoitus havaittiin olevan lisämekanismi sytokiinien aktivoiman Jak2:n negatiiviseen säätelyyn. Jotta Jak2-proteiini ubiquitinisoituisi sen täytyy olla fosforyloitunut. Myös suppressor of cytokine signaling 1:n (SOCS1) havaittiin osallistuvan Jak2:n ubiquitinisaation.

Tämän tutkimuksen pohjalta näyttää todennäköiseltä, että sekä ubiquitinisaatio että PKC-δ välitteinen Jak2:n esto toimivat molemmat sytokiinisignaloinnin hienosäätelijöinä.

2. LIST OF ORIGINAL COMMUNICATIONS

I P.E. Kovanen, I. Junttila., K. Takaluoma, P. Saharinen, L. Valmu, W. Li and O. Silvennoinen: Regulation of Jak2 tyrosine kinase by protein kinase C during macrophage differentiation of IL-3-dependent myeloid progenitor cells, *Blood*, 2000 March;95(5):1626-1632

II I. Junttila, P.E. Kovanen, Q. Xu, P. Mäntymaa and O. Silvennoinen: FcγR inhibits IFN-γ signaling through PKC-δ activation. Submitted for publication

III I. Junttila, R. P. Bourette, L.R. Rohrschneider and O. Silvennoinen: M-CSF-induced differentiation of myeloid precursor cells involves activation of PKC-δ and expression of Pkare, *J Leukoc Biol*, 2003 February;73(2):281-288

IV D. Ungureanu, P. Saharinen, I. Junttila, D.J. Hilton and O. Silvennoinen: Regulation of Jak2 through the Ubiquitin-Proteasome pathway involves phosphorylation of Jak2 on Y1007 and interaction with SOCS-1, *Mol Cell Biol*, 2002 May ;22(10):3316-3326

Original communication number IV will also be published in Academic dissertation of Daniela Ungureanu.

3. ABBREVIATIONS

APC	Antigen presenting cell
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
C	Carboxy, COOH
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CIS	Cytokine-inducible Src homology 2-domain containing protein
CRE	cyclic adenosine monophosphate response element
CSF	Colony stimulating factor
DMEM	Dulbecco's mofified Eagle's medium
EMSA	Electrophoretic gel mobility assay
EPO	Erythropoietin
ERK	Extracellular regulated kinase
FACS	Fluorescence activated cell sorter
FcγR	Fcγ receptor
FCS	Foetal Calf serum
FITC	Fluorescein isothiocyanate
GAS	Interferon-γ-activated sequence
GM-CSF	Granulocyte-macrophage colony stimulating factor
HA	Hemagglutinin
H_2O_2	Hydrogen peroxide
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRF	Interferon regulatory factor
ISRE	IFN-α-stimulated response element
ITAM	Intracellular immunoreceptor tyrosine-based activation-domain
ITIM	Intracellular immunoreceptor tyrosine-based inhibition-domain
JAK	Janus kinase
kDa	Kilodalton
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
M-CSF	Macrophage colony-stimulating factor
MEK	Mitogen-activated ERK-activating kinase

MG132	carbobenzoxyl-L-leucyl-L-leucyl-L-leucinal; Z-LLL-CHO, proteasome inhibitor
MHC	Major histocompatibility complex
mRNA	messenger RNA
N	Amino, NH2
NO	Nitric oxide
O_2^-	Superoxide anion
PBS	Phosphate buffered saline
PD098059	2'-Amino-3'-methoxyflavone, a MEK/ERK inhibitor
PI-3K	Phosphoinositide-3-kinase
PIAS	Protein inhibitor of activated STAT1
PKA	Protein kinase A
Pkare	Protein kinase A related kinase
PKC	Protein kinase C
PLC	Phospholipase C
PMSF	Phenylmethylsulfonyl fluoride
PRKX	Protein kinase X
PTP	Protein tyrosine phosphatase
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SH-2	Src homology-2
SHP	SH-2 domain containing phosphatase
SOCS	Suppressor of cytokine signaling
STAT	Signal transducer and activator of transcription
SUMO	Small ubiquitin-like protein modifier
TPA	12-O-tetradecanoyl-phorbol-13-acetate
X-SCID	X-linked severe combined immunodeficiency

4. INTRODUCTION

Extracellular conditions vary constantly in response to physiological changes in the body and tissues. Therefore cells have to respond appropriately to the varying conditions in order to maintain physiological homeostasis. Information from extracellular space is transduced to the nucleus, where alterations in gene expression bring about the required changes in cell functions. Inappropriate responses to extracellular signals may result in malignant cell growth or increased apoptosis. An important mechanism for signal transduction is receptor-mediated signaling. Hematopoiesis is an excellent example of a precisely regulated cellular event, where the expression of hematopoietic cytokine receptors is induced by a set of critical transcription factors, resulting in responsiveness to certain cytokines, and ultimately leading to lineage commitment (Clarke and Gordon 1998).

Myelopoiesis is a process resulting in the production of new myeloid cells from myeloid precursor cells in the bone marrow. IL-3 is a cytokine with broad target specificity in supporting the production of macrophages, neutrophils, eosinophils, basophils, mast cells, megakaryocytes and erythrocytes by supporting the growth of pluripotent hematopoietic stem cells and myeloid precursor cells (Barreda et al. 2004). IL-3 is not necessary only for early progenitors: terminally differentiated macrophages also require IL-3 for survival and anti-apoptosis. M-CSF is the only cytokine supporting macrophage differentiation uniquely, and M-CSF has been shown to inhibit IL-3-induced proliferation of differentiating macrophages (Bourette et al. 1995).

Macrophages are the effector cells of innate immunity with important functions in phagocytosis and cytokine expression. Several mechanisms have evolved to ensure macrophage activity, for example, T-cell-mediated activation via the expression of IFN-γ, and B-cell-mediated activation through receptors for the constant domain of B-cell-expressed immunoglobulins (FcR).

Since the immediate signaling of most cytokines is fairly well-characterized, more effort has been put on understanding the negative regulation of cytokine signaling. Several mechanisms of the feedback inhibition of cytokine signaling have been described during the past ten years (Wormald and Hilton 2004). However, it appears that the negative regulation of signaling consists of mechanisms with overlapping functions working in finely-tuned balance. By understanding better how the negative regulation of hematopoietic cytokines is achieved, specific targets for future treatments against cancer and autoimmune diseases can be discovered. This study was set up to investigate the mechanisms of the negative regulation of cytokines important in macrophage growth and

activation (IL-3 and IFN-γ). Additionally, the signaling of M-CSF, a negative regulator of IL-3-induced growth signaling, was studied.

5. REVIEW OF THE LITERATURE

5.1. Macrophages

Macrophages are the key components of the first line of defense in the immune system. Macrophages were initially described by L. Aschoff in 1924 as part of the reticulo-endothelial system. The reticulo-endothelial system consists of cells which clear apoptotic and tumour cells and tissue debris from the blood through phagocytosis. The main macrophage function is indeed phagocytosis, but in addition, macrophages secrete small glycosylated proteins, cytokines, which have important regulatory effects on immune responses, hematopoiesis and inflammatory responses (Shepard and Zon 2000). Macrophage maturation from bone-marrow pluripotent self-renewing stem cells into mature tissue macrophages occurs via several differentiation steps.

Macrophages are widely distributed in the body either freely or residentially in different tissues, organs, and body cavities (Shepard and Zon 2000). Due to this wide distribution, the functions and morphology of body macrophages can vary substantially. The functions macrophages regulate are numerous: defense against microorganisms and tumor cells, the regulation of the inflammatory and immune responses and the regulation of hematopoiesis and wound healing (Ginsel 1993).

Macrophages are well conserved evolutionarily. Despite the lack of adaptive immunity, *Drosophila melanogaster*, the fruit fly, has circulating macrophages which are capable of phagocytosis similarly to mammalian macrophages. The signaling pathways leading to macrophage activation are highly conserved from *Drosophila* to humans (Hoffmann and Reichhart 2002). The activity of macrophages is regulated in several different ways (Gordon 2003, Mosser 2003). Depending on the activation signal slightly different cellular outputs will ensue. The mechanisms of macrophage activation and the resulting functional changes will be briefly discussed next.

Innate macrophage activation occurs via pattern recognition receptors, when receptor recognizes a non-self or altered-self structure such as lipopolysaccharide (LPS), peptidoglycans, bacterial CpG DNA or apoptotic/necrotic host-cells. Subsequently macrophages enhance their ability to phagocytose and the expression of toxic products increases in order to destroy intracellular non-self or altered-self particles. These toxic products include hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and nitric oxide (NO) (Gordon 2003).

Humoral activation is elicited by Fc and complement receptors upon the binding of a corresponding ligand to the receptor. The cytolytic activity of macrophages is increased in response to humoral activation. Additionally, the secretion of either pro- or anti-inflammatory cytokines is increased (Gordon 2003).

Classical activation results from Intereferon-γ (IFN-γ) binding to its cognate receptor simultaneously with LPS leading to the expression of pro-inflammatory cytokines (IL-1 and IL-6), reactive oxygen species (ROS) and NO. In addition, the expression of major histocompatibility class II (MHCII) is increased and thus the ability of a macrophage to act as an antigen presenting cell (APC) is induced (Mosser 2003).

An alternative activation of macrophages results from IL-4 and IL-13 stimulation. These cytokines are expressed in allergic, parasitic and tissue repair responses. Alternative activation leads also to the induced expression of MHC II and antigen endocytosis to ensure that T-cells have free access to intracellular antigens (Gordon 2003).

According to recent findings, an additional activation route for macrophages may still exist, which is termed type II activation. In this type II activation, Fc receptor ligation combined with an additional signal results in an activation pattern similar to classical activation with the exception of large amounts of IL-10 being produced in the absence of IL-12 production (Sutterwala et al. 1997, Sutterwala et al. 1998). IL-10 is a potent deactivator of macrophages and it also increases IL-4 production from T-cells leading to T helper type 2 (Th_2) responses. Thus it could be envisaged that type II activation of macrophages might actually be an anti-inflammatory response.

5.2. Hematopoiesis

Hematopoiesis is a continuous process where new blood cells are constantly produced to replace senescent cells. In adults, hematopoiesis occurs in the bone marrow, where a limited number of self-renewing, pluripotent stem cells differentiate into populations of lineage-committed common myeloid and common lymphoid precursor cells. Myeloid precursor cell gives rise to monocytes, erythrocytes, granulocytes and megakaryocytes, and a lymphocyte precursor cell to B and T-cells (Ogawa 1993). Pluripotent stem cells either self-renew and maintain stem cell properties or give rise to cells increasingly committed to differentiation. It is still unclear how the decision between differentiation and proliferation in stem cells is made. However it appears that a specific set of transcription factors regulates the lineage commitment by inducing the expression of cell-type-specific genes but the mechanism of transcription factor regulation is still unknown (Skalnik 2002). Though it has been suggested that the microenvironment *per se* could direct cells towards differentiation or proliferation (Socolovsky et al. 1998), it appears that the fate of

the cell is a stochastic program, and the microenvironment in the bone marrow consisting of soluble factors such as cytokines and cell-cell and cell-ECM (extra-cellular matrix) interactions only reflects the needs of a predestinated cell (Goldsmith et al. 1998, Stoffel et al. 1999). Thus, a stochastically expressed combination of growth factor receptors appears to play a critical role in the commitment of a stem cell into a certain direction of differentiation.

5.3. Overview of factors that regulate macrophage differentiation and function

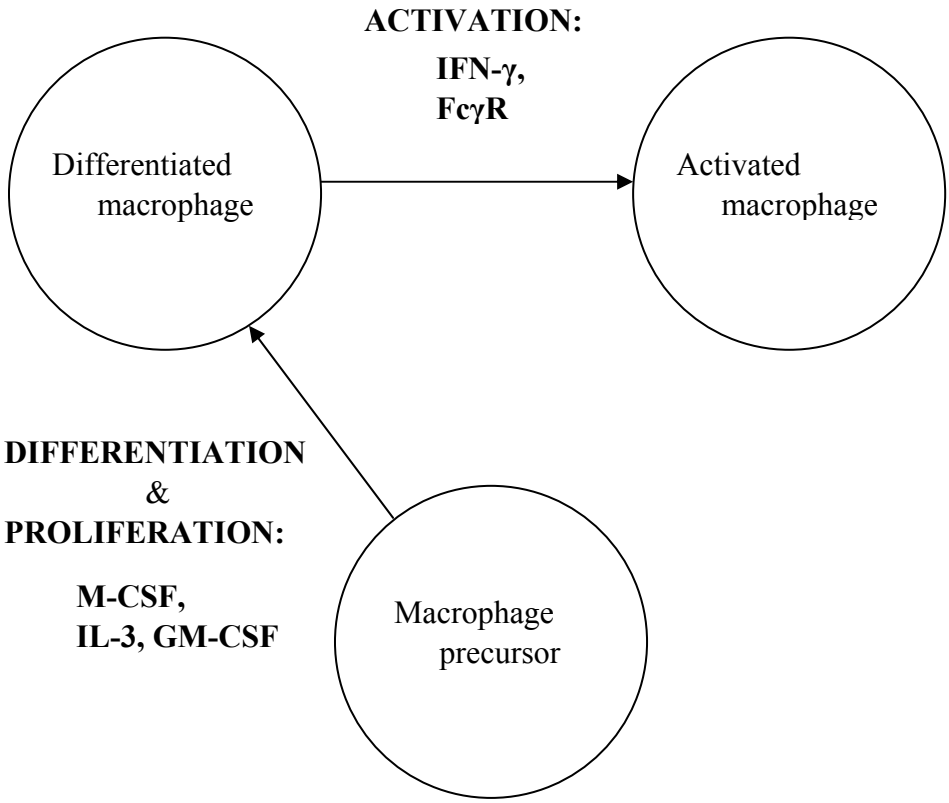

Figure 1. Schematic presentation of factors regulating macrophage differentiation and activation.

5.3.1. Genes involved in the regulation of myeloid differentiation

Precise lineage-specific and differentiation-stage specific gene expression is important for hematopoiesis (Clarke and Gordon 1998). Transcription factors

have a key role in regulating the expression of genes involved in myeloid differentiation. Early stages of myeloid differentiation require the expression of transcription factors regulating survival (GATA-1, GATA-2, PLZF and SCL) and early differentiation (c-myb, c-myc and C/EBPα), and their expression decreases upon terminal differentiation (Valledor et al. 1998). PU1 and AML1 are myeloid-specific transcription factors that are constantly expressed, whereas the expression of c-Jun, CEBPα and Egr1 is increased during myeloid differentiation. Myeloid-specific transcription factors co operate in the regulation of gene expression with each other and also with non-myeloid-restricted transcription factors. Eventually, genes that are regulated by these critical transcription factors give rise to fully-differentiated effector cells (Valledor et al. 1998, Zhu and Emerson 2002).

5.3.1.1. PRKX as an example of myeloid differentiation-associated gene

Protein kinase X (PRKX) is a recently discovered gene that has been characterized as a serine/threonine kinase (Schiebel et al. 1997). PRKX regulates myeloid differentiation as well as the development of kidney morphology (Semizarov et al. 1998, Li et al. 2002). PRKX gene is located in the human X chromosome at Xp22.3. PRKX resembles the catalytic subunit of protein kinase A (PKA), and PRKX probably activates PKA-sensitive pathways (Zimmermann et al. 1999). Early reports suggesting ubiquitous PRKX expression in tissues were probably biased by blood contaminations in tissue samples, because it was later shown that RNA purification by total RNA method instead of poly(A)$^+$ RNA purification did not yield PRKX expression outside blood cells (Klink et al. 1995, Semizarov et al. 1998). However, Li et al. did find PRKX expression in kidneys and they show PRKX to play a role in kidney morphognesis (Li et al. 2002), and quite recently PRKX expression was shown to be ubiquitous during embryogenesis, but more restricted in adulthood as determined by protein level measurements (Li et al. 2005).

PRKX is required for myeloid differentiation, and it is expressed in monocytes, granulocytes and macrophages, but not in immature myeloid cells or lymphocytes. This indicates a definitive role for PRKX in myeloid differentiation. This is further emphasized by impaired myeloid differentiation in the cells where functional expression of PRKX was inhibited by antisense oligonucleotides. In myeloid differentiation signaling, upstream regulators of PRKX include PKC-β, but the downstream targets of PRKX still remain elusive (Semizarov et al 1998).

PRKX is evolutionarily conserved, and its highly homologous *Drosophila* gene (DC2) is important during *Drosophila* embryogenesis. However, the genetically modified *Drosophila* lacking functional DC2 was viable, which suggests a specific and limited role for DC2 in *Drosophila* development (Melendez et al.1995). In a mouse, a homologue for PRKX has been described and termed Protein kinase A related kinase (Pkare) (Blaschke et al. 2000). Pkare

was identified with degenerative primer method, and as opposed to PRKX expression in humans it is widely expressed in adult mice. Antisense in situ hybridization was used to define Pkare expression during mouse embryogenesis, and Pkare expression was restricted to differentiating neurons (Blaschke et al. 2000). However, there is still only a limited amount of knowledge about the role of Pkare in mouse physiology.

5.3.2. Signaling of cytokines which regulate macrophage differentiation

Differentiation of macrophages is precisely coordinated by a network of hematopoietic cytokines and polypeptide growth factors. The term cytokine by definition refers to soluble factors regulating cellular functions (Silvennoinen and Ihle 1996). The word cytokine is used in this study to refer to soluble factors regulating hematopoietic cell functions through specific transmembrane receptors. Hematopoietic cytokines are a group of small secreted glycoproteins. Cytokines transfer information from the extracellular environment into cells through transmembrane receptors. Receptors transmit information into cellular responses through various mechanisms, the most important being protein phosphorylation. Protein phosphorylation on tyrosine residues covers only 0,5 % of total cellular phosphorylation (Schlessinger and Ullrich 1992) but it is a crucial regulatory mechanism of most cell functions. Several cytokines and polypeptide growth factors are important in macrophage differentiation, particularly IL-3, granulocyte-macrophage colony stimulating factor (GM-CSF), and M-CSF.

5.3.2.1. The mechanisms of cytokine receptor signaling

Cytokine receptors can be roughly divided into five subcategories depending on the number of receptor subunits and/or the utilization of shared subunits. These include single chain receptors (e.g. Epo, Tpo, GH, PRL), common β-chain receptors (IL-3, IL-5, GM-CSF), gp130 family (IL-6, IL-11, CNTF, LIF, OSM, IL-12), the common γ-chain family (IL-2, IL-4, IL-7, IL-9, IL-13, IL-15) and interferon receptors (IFN-α, -β, -γ and IL-10) (Ihle et al. 1995). In addition to cytokines, hematopoiesis is regulated by polypeptide growth factors like M-CSF and stem cell factor (SCF). The cytoplasmic domain of cytokine receptors does not contain intrinsic catalytic activity whereas polypeptide growth factor receptors such as M-CSF are receptor tyrosine kinases (RTK) (Ihle et al. 1995) and they will be discussed later. In the case of cytokine receptors, the discovery of IFN-responsive Stat transcription factors led to the characterization of novel signal transduction pathway utilizing Stat and Jak kinases (Darnell et al. 1994).

5.3.2.2. Jaks

Four members of the Jak family have been identified (Jak1-3 and Tyk2) (Leonard and O'Shea 1998). Jaks are relatively large in molecular size: 120-130 kDa. One Jak kinase, which is termed *hopscotch,* exists in *Drosophila melanogaster*. Jaks contain seven distinguished conserved domains: Jak homology domains (JH) 1-7. The unique structure in Jaks is a tandem kinase where C-terminal JH1 (kinase domain) is followed by the JH2 (pseudokinase) domain (Kisseleva et al. 2002). JH1 is the catalytically active domain of Jak, whereas JH2 is catalytically inactive. Interestingly, the JH2 domain of Jak2 and Jak3 autoregulates its kinase activity (Saharinen and Silvennoinen 2002), and a single mutation in Jak2 JH2 has recently been shown to be involved in the pathogenesis of polycythemia vera (James et al. 2005, Levine et al. 2005). The relatively divergent JH3-JH7 domains appear to regulate interactions with cytokine receptors, which allows specificity for Jak/receptor binding (Leonard and O'Shea 1998, Ihle 2001). Jak3 domains JH7-JH5 contain a band four-point-one, ezrin, radixin, moesin (FERM) homology domain (Girault et al. 1999). The FERM domain mediates Jak interactions with transmembrane proteins, such as cytokine receptors, and positively regulates catalytic activity by binding to JH1 domain (Yamaoka et al. 2004).

Jak1 is widely expressed in tissues. Jak1 is important in signaling through common γ-chain receptors, gp130 receptors and IFN receptors (Kisseleva et al. 2002). Due to various vital functions mediated by these cytokine receptors, a Jak1 knock-out mouse dies postnatally due to neurological defects and also suffers from lymphopenia (Rodig et al. 1998).

Ubiquitously expressed Jak2 regulates signaling through single chain receptors, common β-chain receptors and IFN receptors (Schindler and Strehlow 2000). Jak2 deficiency causes even more severe phenotype than Jak1 deficiency, probably resulting from deficient erythropoiesis due to dysregulated erythropoietin (EPO) receptor signaling. A Jak2-deficient mouse dies at day 12,5 of gestation (Neubauer et al. 1998).

Jak3 expression is restricted to hematopoietic cells (Leonard and O'Shea 1998). Jak3 is an important regulator of signaling by cytokines which utilize common γ-chain. The phenotype of a genetically modified mouse which lacks functional Jak3 (X-linked severe immunodeficiency; X-SCID) is not lethal. However, the mouse has defective lymphopoiesis of both T- and B-cells whereas a human lacking functional Jak3 only has defective T-cell population. This difference is probably due to the requirement of IL-7, a common γ-chain cytokine for pre B-cells in mice but not in humans (Nosaka et al. 1995, Thomis et al. 1995, Kisseleva et al. 2002).

Widely expressed Tyk2 was the first identified member of the Jak family (Krolewski et al. 1990). The receptors which signal through Tyk2 include IFN-α, IL-6, IL-10 and IL-12 (Kisseleva et al. 2002). Quite unexpectedly, a Tyk2 deficient mouse had almost normal IL-6 and IL-10 responses, defective IL-12

signaling and dose-dependent IFN-α unresponsiveness (Karaghiosoff et al. 2000).

5.3.2.3. Stats

The Stat family of transcription factors consists of seven members in mammals, Stat1-4, Stat5a and Stat5b and Stat6. *Drosophila melanogaster* has one Stat gene (Stat92E) as well as one Jak gene operating in the same signal transduction pathway which indicates that evolutionarily the Jak/Stat pathway is conserved (Hou and Perrimon 1997). Six conserved domains are shared by seven mammalian Stats. These include C-terminal transactivation domain (TAD), SH-2 domain, linker domain, DNA-binding domain, coiled-coil domain and N-terminal domain (Paukku and Silvennoinen 2004). Stat activation occurs upon cytokine binding to its cognate receptor which induces receptor-associated Jaks to tyrosine phosphorylate receptor chains. Tyrosine phosphorylated receptor chains then serve as docking sites for the SH2 domain of Stats. Stats become tyrosine phosphorylated upon receptor chain binding. Tyrosine phosphorylation induces Stat dimerization which leads to the detachment of Stat from the receptor chain and its translocation to the nucleus where Stat dimers bind DNA at specific sites with their DNA-binding domain and regulate gene transcription through their trans-activation domain (Darnell et al. 1994, Ihle et al. 1995, Horvath and Darnell 1997). The classical view on Jak-Stat signaling has been somewhat modified. Recently it was shown that Stat tyrosine phosphorylation is not absolutely necessary for Stat dimerization (Braunstein et al. 2003).

Stat1 and Stat2 were initially described in IFN signaling because IFN-α and –β were discovered to induce homodimerisation of Stat1 and heterodimerisation of Stat1/Stat2 which in association with the IRF-9 (p48) protein create a complex (IFN-stimulated gene factor-3; ISGF-3) regulating IFN-stimulated response element (ISRE) driven genes. IFN-γ in turn induces the homodimerisation of Stat1 and the binding of newly formed homodimers to gamma activated sequence (GAS) (Fu et al. 1992, Shuai et al. 1992).

Stat3 was originally identified as an IL-6 induced factor, but it is activated by several cytokines. Stat3 affects cell proliferation either by up-regulating or down-regulating proliferation in a cell-type-specific manner (Paukku and Silvennoinen 2004).

Stat4 is the only Stat with relatively limited expression in myeloid cells, thymus, testis, (Zhong et al. 1994) natural killer (NK) cells, dendritic cells and T-lymphocytes (Paukku and Silvennoinen 2004). In T-cells Stat4 promotes IL-12-mediated Th_1 differentiation (Usui et al. 2003). In antigen presenting cells (APCs) Stat4 mediates IFN-γ expression, an important cytokine for Th_1 differentiation (Frucht et al. 2001).

Stat5a and Stat5b are closely related genes, sharing over 90% of their sequence (Darnell 1997). Stat5a was initially characterized in prolactin (PRL) signaling, as PRL induced a transcription factor binding the β-casein promoter

(Wakao et al. 1994). Despite sequence homology Stat5a and Stat5b appear to regulate different functions, as described by Stat5a $^{-/-}$ and Stat5b $^{-/-}$ mice as well as double knock-out mice lacking both functional Stat5a and Stat5b (Paukku and Silvennoinen 2004). The deletion of Stat5a results in decreased PRL signaling and impaired mammary gland development, whereas Stat5b deletion leads to dysregulated growth hormone (GH) signaling and growth retardation (Liu et al. 1997, Udy et al. 1997). The deletion of both Stat5a and Stat5b results in infertility, growth retardation, impaired mammary gland development, lymphopenia and hypocellular bone marrow (Miyoshi et al. 2001). Stat5 appears to be involved in malignancies such as acute myeloid and acute lymphoid leukemia, *in vivo* (Weber-Nordt et al. 1996, Gouilleux-Gruart et al. 1997, Yu et al. 1997) as well as *in vitro* in IL-3-dependent Ba/F3 and 32D cells (Onishi et al. 1998, Nieborowska-Skorska et al. 1999).

Stat6 was initially identified as an IL-4 induced transcription factor binding GAS (Kotanides and Reich 1993). In addition to IL-4, Stat6 regulates signaling through IL-13. Both of these cytokines are important in adaptive immunity. Mice lacking functional Stat6 have defects in Th_2 cell differentiation. Interestingly, the deletion of Stat6 has a more severe effect on Th_2 differentiation than the deletion of IL-4, due to defects in IL-13 signaling (Kisseleva et al. 2002).

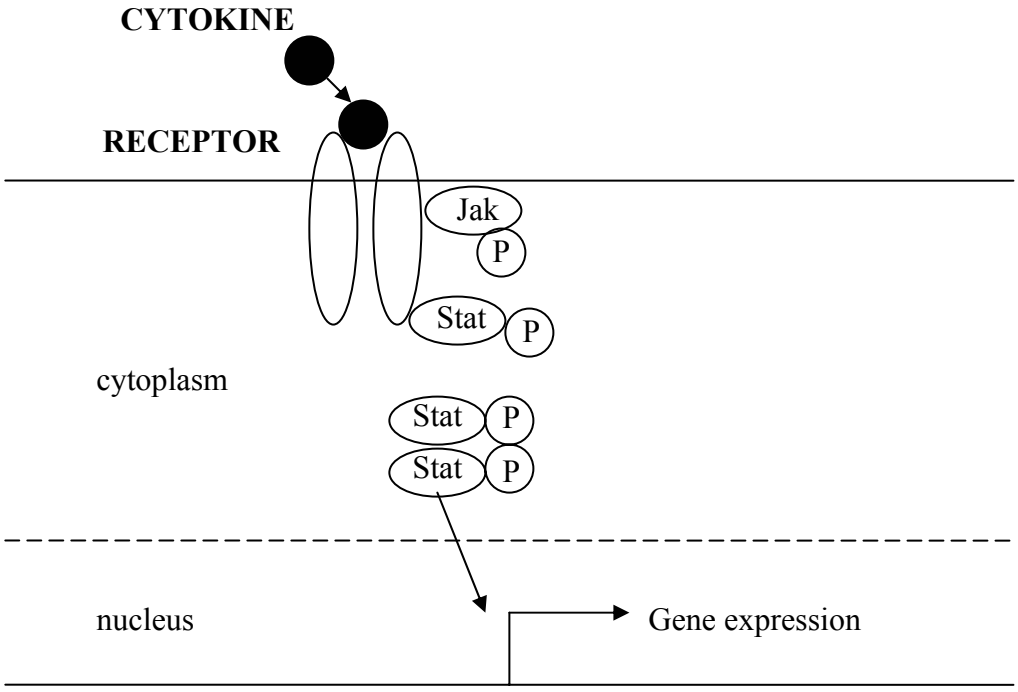

Figure 2. An overview of cytokine signaling. The binding of a cytokine to its cognate receptor activates receptor-associated Jaks and subsequently Stats which translocate to the nucleus and bind specific areas of DNA, and thus regulate gene expression.

5.3.2.4. GM-CSF

In combination with other cytokines, Granulocyte-macrophage colony-stimulating factor (GM-CSF) promotes the maturation and growth of macrophages, eosinophils, and neutrophils and supports the growth and development of erythroid and megakaryocyte progenitors. GM-CSF has pleiotropic effects on hematopoietic cells and overlapping functions with other cytokines, including IL-3, IL-6, G-CSF and M-CSF (Barreda et al. 2004). In contrast with IL-3, GM-CSF is expressed and secreted from various cells including fibroblasts, endothelial cells, B- and T-lymphocytes, macrophages, eosinophils, neutrophils and mast cells, to name a few (Gasson 1991). In humans, GM-CSF is a glycoprotein of 14-33 kDa, depending on the amount of the glycosylation of the protein. GM-CSF exerts its functions through a heterodimeric α/β receptor, where β-chain is the common β-chain shared by the IL-3, IL-5 and GM-CSF receptors (Bagley et al. 1997). α-chain is cytokine-specific, and by itself it has a low ability for cytokine binding. The binding of the ligand to the α-chain induces α and β-chain dimerization, the formation of high affinity receptors and the induction of intracellular signaling. In the GM-CSF receptor, the Jak2 kinase is constitutively associated with the membrane proximal part of β-chain, and receptor-chain associated Jak2 molecules can cross phosphorylate each other and the receptor β-chain upon receptor chain

dimerization (Quelle et al. 1994). Jak2 activation in turn leads to Stat5 binding to the receptor, phosphorylation, homodimerisation, and nuclear translocation/DNA binding (Leake 1999) as discussed in detail in the context of IL-3. Interestingly, the α-chains of IL-3 and GM-CSF receptors appear to have a common ancestral gene (Nakagawa et al. 1994, Kosugi et al. 1995). GM-CSF and IL-3 have overlapping functions on progenitor cells. However, IL-3 appears to target the earliest progenitors whereas GM-CSF regulates slightly more differentiated cells (Koike et al. 1987). The regulation of GM-CSF-mediated functions can also be obtained through varying cytokine concentrations. A 10-fold amount of GM-CSF is required to stimulate erythroid and megakaryocytic colony formation compared to granulocytic or macrophage colony formation (Barreda et al. 2004). In addition, the treatment of granulocyte-macrophage progenitors with a high concentration of GM-CSF in combination with M-CSF results in granulocyte formation, but a low concentration of GM-CSF favors macrophage differentiation (Metcalf 1980).

5.3.2.5. IL-3

IL-3 (also known as multi-colony-stimulating factor, multi-CSF) is a growth and survival factor for cells of the myeloid lineage. IL-3 is a disulfide-bridged glycoprotein of 14-30 kDa (size variation mainly due to glycosylation) in humans, and it is produced mainly by Th cells which thus support the growth of immature hematopoietic cells. Th-restricted expression of IL-3 suggests that the role of IL-3 is to support hematopoiesis and stem cell differentiation during immune response (Ihle 1992). The lack of IL-3 expression in mice has no immediate effect on hematopoiesis (Nishinakamura et al. 1996) whereas the administration of IL-3 up-regulates hematopoiesis (Metcalf et al. 1986). IL-3 exerts its functions through a transmembrane cytokine receptor on the cell surface. The receptor consists of two receptor chains; α-chain and β-chain. As discussed above, β-chain is shared by the IL-3, IL-5 and GM-CSF receptors which together form a subfamily of the IL-3 family of cytokines. The activation of the IL-3 receptor occurs when IL-3 binds with low affinity to α-chain (molecular weight 41 kDa) which in turn induces the dimerization of α- and β-chains. Itself, β-chain (mw 130 kDa) does not bind IL-3, but when it associates with α-chain, a high affinity cytokine receptor is formed. α-chain is cytokine-specific and it is responsible for the recognition of the cytokine. β-chain activation in turn is mainly responsible for intracellular signaling events elicited by these receptors (Barreda et al. 2004). Receptor chain dimerization induces rapid phosphorylation of intracellular common β-chain tyrosine residues, and these phosphorylated residues then serve as docking sites for signaling molecules that contain the SH-2 domain (de Groot et al. 1998). The intracellular common β-chain is approximately 450 amino acids long and different parts of this intracellular domain contribute to various cellular functions such as proliferation and cell-survival (Itoh et al. 1998). Several molecules recruited to the

phosphorylated IL-3 receptor β-chain have been identified but their role in IL-3 signaling is poorly understood. These include Lyn, Btk, Tec, Fyn and Hck. Jak2 and Stat family members are also activated by IL-3. Stat5a and Stat5b are strongly activated by IL-3/IL-5/GM-CSF receptors. These receptors also activate other Stat family members such as Stat1, Stat3 and Stat6, depending on the cell type studied (de Groot et al. 1998). Stat5 activation results from tyrosine phosphorylation by Jak2 as Stat5 is associated with the receptor β-chain. It is of interest though, that the C-terminal deletion mutant of the IL-3 receptor β-chain is capable of activating Jak2 but incapable of activating Stat5, indicating that Stat5 activation is not regulated only by Jak2 (Smith et al. 1997). Docking sites in the IL-3 receptor β-chain are important for Stat5 activation, since the mutation of four tyrosines Y577, Y612, Y695 and Y750 completely blocks IL-5-induced Stat5-mediated transcription (van Dijk et al. 1997). Stat5 tyrosine phosphorylation leads to the homodimerization and nuclear translocation of Stat5. In the nucleus Stat5 binds to DNA and thus regulates the expression of specific set of genes (Larner et al. 1993).

5.3.2.6. Receptor tyrosine kinases (RTKs)

As mentioned above, the fundamental difference between polypeptide growth factors and hematopoietic cytokines is the intrinsic kinase activity of the receptor, which is not found in the receptors of hematopoietic cytokines. Polypeptide growth factor receptors contain intrinsic tyrosine kinase activity, and they are termed Receptor tyrosine kinases (RTK). A ligand binding to RTK induces the dimerization of the receptor chains and the tyrosine phosphorylation of the receptor chains which in turn tyrosine phosphorylate intracellular signaling molecules (Weiss and Schlessinger 1998). RTKs contain an extracellular ligand binding domain, a transmembrane domain, and a cytoplasmic domain which contains the protein tyrosine kinase core flanked by regulatory sequences (Hubbard and Till 2000).

5.3.2.7. M-CSF

Macrophage-colony stimulating factor (M-CSF or CSF-1) regulates the proliferation, activation and differentiation of monocyte/macrophage-committed hematopoietic cells. M-CSF is a homodimeric glycoprotein with a molecular weight ranging from 44 to 150 kDa (Fixe and Praloran 1997, Motoyoshi 1998). As with all the colony stimulating factors, the name of the cytokine is derived from its ability to promote the macrophage colony formation of mouse bone marrow cells in a semisolid agar culture system. M-CSF is expressed by number of cells, including endothelial cells, astrocytes, myoblasts, fibroblasts, bone marrow stromal cells, thymic epithelial cells and endometrial gland cells

(Barreda et al. 2004). Activated B- and T-cells, macrophages and fibroblasts can also produce M-CSF.

The receptor for M-CSF (Fms) is encoded by proto-oncogene *c-fms* and it belongs to the family of RTKs discussed above. The receptor is a 140-150 kDa homodimer. The intracellular domains of the receptor include juxtramembrane region, kinase insert (KI) region, tyrosine kinase (TK) region, and C-terminus. The binding of M-CSF to Fms brings about receptor chain homodimerization which leads to the increased kinase activity of Fms kinase domains. Fms kinase domain activation in turn leads to the phosphorylation of critical tyrosine residues of the intracellular domains of Fms. Phosphorylated tyrosine residues in the receptor itself then serve as docking sites for signaling molecules that contain SH-2 domain (Hamilton 1997, Pixley and Stanley 2004). Several signaling cascades are activated by Fms activation and the autophosphorylation of critical tyrosine residues. At least five different tyrosine residues in Fms bind and activate individual signaling molecules. In murine Fms, tyrosine 559 (juxtamembrane region) binds and activates Src family members (Marks et al. 1999), tyrosine 697 (KI region) activates GRB2 (van der Geer and Hunter 1993), tyrosine 706 (KI region) activates Stat1 (Novak et al. 1996), tyrosine 721 (KI region) activates PI 3'-kinase (Reedijk et al. 1992), tyrosine 807 (TK domain) activates Phospholipase Cγ-2 (PLCγ2) (Bourette et al. 1997) and tyrosine 973 (C-terminus) activates c-Cbl (Wilhelmsen et al. 2002). The introduction of point mutations of these tyrosine residues, followed by the functional characterization of cells expressing these mutants has revealed that a single tyrosine residue, Y807 is critical for Fms-mediated differentiation even though other tyrosine residues facilitate the differentiation process (Bourette et al. 1995).

5.3.3. Factors that regulate macrophage functions

As described above (pp. 15-16), macrophages are functionally responsive to several types of stimuli from endogenous and exogenous sources. The most important cytokine regulating macrophage activity is Interferon-γ (IFN-γ) expressed in T-cells during immune responses. In addition, during allergic responses and parasitic infections cytokines IL-4 or IL-13 can activate macrophages. Non-cytokine-mediated macrophage activation includes the recognition of foreign structures by specific receptors on the macrophage cell surface (pattern recognition receptors). These include receptors against bacterial DNA, LPS and peptidoglycans. Also, the Fc domains of immunoglobulins and members of complement system have their own receptors on the macrophage cell surface (Gordon 2003). Fc receptors also play an important role in macrophage activation leading to IL-10 production and anti-inflammatory responses.

5.3.3.1. IFN-γ

A key regulator of macrophage activation is IFN-γ. IFN-γ is a noncovalent homodimer which contains two identical 17 kDa polypeptide chains (Gray et al. 1982) which are variably N-glycosylated (Bach et al. 1997). In addition to macrophage activation, IFN-γ regulates immune responses more widely. It promotes the differentiation of naïve T-cells into Th_1-type cells and induces Ig isotype switching in B-cells. It also promotes the production of important antimicrobial cytokines such as the p40 subunit of IL-12, Tumor necrosis factor (TNF)-α and IL-1 (Decker et al. 2002). IFN-γ itself regulates the expression of probably hundreds of genes (Ramana et al. 2002), and the elucidation of the signaling mechanisms elicited by the IFN-γ receptor has provided a comprehensive molecular understanding of the events leading to IFN-γ-induced cellular responses. IFN-γ is expressed mainly by the cells of the immune system: natural killer cells (NK), Th_1-cells and cytotoxic T-cells. In addition, macrophages and activated dendritic cells produce IFN-γ in the presence of IL-12 and IL-18 (Shtrichman and Samuel 2001). IFN-γ expression is induced by T-cell receptor activation in T-cells and by macrophage-derived cytokines (such as TNF-α, IL-12 and IFN-γ itself) in NK cells (Boehm et al. 1997). The IFN-γ receptor consists of the IFN-γR1 (also called α- or CDw119-chain) and the IFN-γR2 (β- or accessory factor-1 chain) chains which are typical only for the IFN-γ receptor. The binding of the homodimeric IFN-γ to two IFN-γ receptor chains γR1 (IFN-γR1) brings about a receptor complex which consists of two IFN-γR1 and two IFN-γR2 chains. Interestingly, ligand binding does not appear to be absolutely necessary for IFN-γ receptor complex formation as it has also been shown that the IFN-γ receptor chains can be preformed before ligand binding (Krause et al. 2002). The intracellular domains of these chains bind Jak1 (IFN-γR1) and Jak2 (IFN-γR2) kinases. Upon receptor aggregation, receptor-chain-bound Jak kinases cross-phosphorylate each other. Intracellular receptor chains are also phosphorylated. Especially important for IFN-γ signaling is Tyr 440 of the IFN-γR1 chain, which serves as a docking site for Src homology 2 (SH2) domain of Stat1 upon phosphorylation. Stat1 is thus recruited to the receptor complex where it is phosphorylated on tyrosine 701 by Jak1 and Jak2 resulting in Stat1 homodimerisation and nuclear translocation. Quite recently it has also been shown that Stat dimerisation may also occur independently of tyrosine phosphorylation (Braunstein et al. 2003). In the nucleus, Stat1 homodimers bind to their target sequences called GAS on DNA, resulting in the regulation of IFN-γ responsive genes (Bach et al. 1997, Pestka et al. 2004). In addition to tyrosine 701 phosphorylation, full Stat1 responses require an additional phosphorylation on serine 727. Mice with point mutation which alters serine 727 into alanine have a higher mortality rate than wild type mice during a *Listeria monocytogenes* infection, but they are resistant to LPS-induced septic shock syndrome probably due to decreased histone deacetylase recruitment (Varinou et al. 2003). The kinase required for Stat1 serine phosphorylation during stress and inflammation

is p38 (Kovarik et al. 1999) and during IFN-γ stimulation Protein kinase C-δ (PKC-δ) (Uddin et al. 2002).

The Jak/Stat signal transduction pathway is by no means the only signaling pathway activated during IFN-γ-induced macrophage activation. However, the role of other signaling pathways in IFN-γ-induced cellular responses remains to be investigated. At least mitogen activated protein kinases (MAPK), Extracellular regulated kinase 1 and 2 (ERK1/2) and Pyk, Fyn of the Src family of tyrosine kinases, adaptor proteins c-Cbl, Vav, Crkl and CRKII and phosphatases SH2 domain containing phosphatase 1 and 2 (SHP-1 and SHP-2) are activated by IFN-γ (Ramana et al. 2002).

5.3.3.2. FcγR as an example of a non-cytokine activator of macrophages

Immunoglobulins (Igs) are produced by activated B-cells during an immune response. There are three ways Igs protect the host against invading microbes. Firstly, antibodies can neutralize the foreign microbes, thus inhibiting their adherence to the host. Secondly, opsonization facilitates the phagocytosis of invaders. Thirdly, the complement system can be activated by microbe-bound Igs (Janeway et al. 2001). The receptors for the Fc domain of Ig on the macrophage cell surface regulate macrophage activation and allow macrophages to respond to products of adaptive immune response. The receptor for the IgG subclass of Igs is Fcγ Receptor (FcγR). FcγR is a group of three classes of membrane glycoproteins. Two of the classes transduce an activating signal into cells (FcγRI and FcγRIII) and one mediates inhibitory signals into cells (FcγRII) (Ravetch and Bolland 2001). Both activating FcγRs contain immunoreceptor tyrosine-based activation motifs (ITAM) (Isakov 1998). The cross-linking of the FcγR extracellular domain upon ligand binding results in the tyrosine phosphorylation of ITAM. Tyrosine phosphorylation is mediated by members of the Src kinase family. Tyrosine phosphorylated ITAM then serves as a docking site for signaling molecules that contain SH2 domain, depending on the cell type and the subtype of particular activating FcγR (Ravetch and Bolland 2001). On the other hand inhibitory FcγRII contains immunoreceptor tyrosine-based inhibitory motif (ITIM) (Bolland and Ravetch 1999). The tyrosine phosphorylation of ITIM leads to the binding and activation of the inhibitory signaling molecule SHIP which then leads to the inhibition of ITAM activation signaling through the hydrolysis of membrane inositol phosphate PIP3 (Ono et al. 1996). Activating FcγRs are expressed on most effector cells in the immune system: monocytes/macrophages, mast cells, eosinophils, NK cells, neutrophils and platelets, but they are absent from B- or T-cells (Ravetch and Bolland 2001). Generally, inhibitory FcγR is expressed in the same cells as activating FcγRs with the exception of FcγRII expression on B-cells. As both inhibitory and activating FcγRs are usually coexpressed, signaling events elicited by the receptors are finely-tuned, and the expression of the receptor molecules mainly decides whether inhibitory or activating signals are transformed into cell

functions (Ravetch and Bolland 2001). Cell functions mediated by activating FcγRs include increased phagocytosis/degranulation and the increased expression of cytokines and the mediators of inflammation. In addition to preventing the previously mentioned cell functions, inhibitory FcγR inhibits B cell receptor (BCR) induced proliferation (Yamanashi et al. 2000).

CYTOKINE	**SOURCE**	**RECEPTOR (SUBCLASS)**	**HEMATOPOIETIC RESPONSE**
GM-CSF	Lymphocytes, endothelial cells, macrophages, neutrophils, eosinophils	GM-CSF receptor (gp140)	Growth and maturation of macrophages, neutrophils, eosinophils
IL-3	Th lymphocytes	IL-3 receptor (gp140)	Growth and maturation of early lymphoid and myeloid cells
M-CSF	Thymic epithelial cells, endothelial cells, bone marrow stromal cells	M-CSF receptor (also known as CD115, *c-fms*) (Class III RTK)	Macrophage growth and differentiation
IFN-γ	NK cells, Th_1 lymphocytes, cytotoxic T-cells	IFN-γ receptor (IFN-γR)	Macrophage activation, Ig class switch, Th_1 differentiation

Table 1. Characteristics of cytokines regulating macrophage differentiation and activation modified from Barreda et al. 2004 and Pestka et al 2004.

5.4. Mechanisms of the negative regulation of cytokine signaling

The critical step in the signaling through most cytokine receptors is the activation of the members of Jak (Jak1-3 and Tyk2) and Stat (Stat1-4, 5a, 5b and Stat6) families (Ihle et al. 1995). As described above, Jaks play an important role in transducing the signal from the cytokine receptor to the transcription factor (Stat) which in turn regulates the expression of a specific set of genes. Uncontrolled activation of cytokine-receptor-induced signaling would lead to inappropriate cellular responses. For example, dysregulated signaling through anti-inflammatory cytokine IL-10 leads to persistent inflammation (Ji et al. 2003)

whereas uncontrolled proliferative signaling by Jak2 results in cytokine independent proliferation (James et al. 2005). The negative regulation of signaling occurs at several points along the signaling cascades (Chen et al. 2004). Different regulatory mechanisms have evolved to ensure the precise regulation of cytokine signaling and cell functions.

5.4.1. Inhibition through the cross-talk of signaling pathways

Exposure to a cytokine elicits signaling events which ultimately lead to changes in gene expression. Re-exposure to the same or to a different cytokine can have synergistic or antagonistic effects on signaling and the gene expression of the initial cytokine. The effect of the second cytokine is cell-, tissue-, and cytokine-specific. For example, pretreatment with IFN-γ induces IFN-α signaling (Darnell et al. 1994) and IFN-γ signaling itself (Hu et al. 2002) through the induction of the expression of signaling molecules which are important in these IFN responses. A well-characterized mechanism of negative regulation of signaling is the induction of the SOCS protein expression as discussed later. Briefly, IFN-γ inhibits IL-4 responses in human monocytes through the induction of SOCS1 protein which in turn inhibits IL-4-induced Stat6 activation (Dickensheets et al. 1999). IFN-γ signaling can be inhibited by IL-10 probably via the induced SOCS3 expression (Ito et al. 1999). The rapid inhibition of signaling can also occur without new protein synthesis. IL-6-induced Stat3 signaling is rapidly inhibited by a group of MAPKs without the synthesis of new RNA or proteins (Sengupta et al. 1998). Protein kinase A (PKA) down-regulates IFN signaling (David et al. 1996). The signal that initiates negative regulation does not necessarily have to be a cytokine, for example, phorbol esters and immunocomplexes negatively regulate IFN signaling (Feldman et al. 1995, Petricoin et al. 1996). Rheumatoid arthritis is an autoimmune disease characterized by constantly activated macrophages - which have decreased responsiveness to the anti-inflammatory IL-10 cytokine - in joint cartilages, causing sustained inflammation and destruction of the joint cartilages. Another feature of rheumatoid arthritis is the circulating IgG complexes. IgG complexes also inhibit IL-10 signaling through a mechanism which involves PKC isoform δ, a mechanism which may partly explain the pathogenesis of rheumatoid arthritis (Ji et al. 2003).

5.4.2. Protein phosphatases

Rapid and reversible protein phosphorylation is a key mechanism for cellular signal transduction. Protein phosphorylation is the most common post-translational mechanism for the regulation of protein activity. Protein phosphorylation is mediated by protein kinases, which add phosphate groups to

proteins, and reversed by counteracting proteins, phosphatases, which dephosphorylate the target proteins (Hunter 1995). Phosphatases can regulate the dephosphorylation of tyrosine, serine and threonine residues via dual-specific phosphatases, serine/threonine residues via serine/threonine phosphatases, or only tyrosine residues via protein tyrosine phosphatase (PTP) (Trowbridge 1991, Stone and Dixon 1994, Barford 1995). Dual-specific phosphatases regulate cytokine-induced MAPK activity (Zhang et al. 2004). PTPs can be divided into transmembrane and non-transmembrane PTPs. The transmembrane PTPs are characterized by two tandem catalytic domains in the intracellular part combined with fibronectin-type III, immunoglobulin or carbonic anhydrase-like structures in the extracellular domain (Matozaki and Kasuga 1996). Non-transmembrane PTPs have one PTP domain and may contain motif structures such as SH-2, PEST sequence or ezrin-like domain (Matozaki and Kasuga 1996).

Jaks and Stats are targets for several phosphatases including SHP1, SHP2, Cluster of differentiation 45 (CD45), Protein-tyrosine phosphatase 1B (PTP1B) and T-cell protein tyrosine phosphatase (TCPTP) (Shuai and Liu 2003). SHP1 and SHP2 are non-transmembrane PTPs.

For IL-3, SHP1 (also called PTP1C, SHPTP1, SHP or HCP) is the most important phosphatase regulating signaling as the lack of this phosphatase results in increased IL-3-induced tyrosine phosphorylation of the receptor. Ectopic SHP-1 expression in turn results in reduced IL-3-induced tyrosine phosphorylation of IL-3 receptor β-chain (Yi et al. 1993). SHP-1 is associated physically with IL-3 receptor β-chain, the c-Kit receptor and the erythropoietin receptor (EpoR). SHP-1 is mainly expressed in hematopoietic cells and it inhibits Jak2, and Jak1 (David et al. 1995, Klingmuller et al. 1995).

SHP-2 which contains SH-2 domain is expressed ubiquitously. It is associated with the inhibition of Jak1 and cytoplasmic Stat5a (Chen et al. 2003). SHP-2 is a negative regulator of IFN signaling (You et al. 1999).

CD45 is a transmembrane phosphatase highly expressed in hematopoietic cells. CD45 is a potent inhibitor of all Jaks (Irie-Sasaki et al. 2001). CD45 is important for both B-cell and T-cell antigen-induced receptor signaling.

PTP1B is a non-transmembrane phosphatase which inactivates Jak2, Tyk2 and cytoplasmic Stat5 (Shuai and Liu 2003). PTP1B has been associated with obesity and diabetes since PTP1B knock-out mice show enhanced sensitivity to insulin (Elchebly et al. 1999), but no immunological consequences for the loss of the function of the PTP1B-gene have been reported.

TCPTP is closely related to PTP1B. It regulates the tyrosine phosphorylation of Jak1, since IFN-γ-induced Jak1 phosphorylation is increased while Jak2 phosphorylation remains unchanged in TCPTP knock-out mice. Despite the close relations of PTP1B and TCPTP, the functions of these phosphatases as determined by knock-out mice are dramatically different. TCPTP knock-out mice have severely impaired B- and T-cell functions as well as defects in hematopoiesis whereas PTP1B knock-out mice do not have any severe immunological defects. It is of interest that TCPTP specifically dephosphorylates

Jak1, Jak3 and Stat1 whereas PTP1B dephosphorylates Tyk2, Jak2 and Stat5 (Shuai and Liu 2003).

5.4.3. SOCS proteins

The family of Suppressor of Cytokine Signaling (SOCS) contains eight members (SOCS1-7 and cytokine-inducible SH2-domain containing protein1 CIS1). SOCS proteins all contain the SH2 domain and a unique C-terminal motif, the SOCS box. SOCSs inhibit cytokine signaling via different mechanisms, some of which have still not been elucidated (Alexander and Hilton 2004).

The first member of the SOCS family to be described was CIS1 (Yoshimura et al. 1995). CIS1 expression is induced by numerous cytokines and polypeptide growth factors, and it inhibits the Stat5-mediated signaling of several cytokines including IL-2, IL-3, growth hormone (GH), EPO, and prolactin (Kovanen and Leonard 1999). CIS1 binds to a Stat5 binding site and thus blocks Stat5-mediated gene responses (Elliott and Johnston 2004). The importance of CIS1 in the regulation of Stat5 is underlined by the fact that Stat5a and Stat5b knock-out mice have a phenotype similar to that of transgenic mice overexpressing CIS1 (Matsumoto et al. 1999).

SOCS2 structure resembles CIS1 (Elliott and Johnston 2004) and has been suggested to inhibit cytokine signaling by the same method as CIS1, but the exact mechanism how SOCS2 negatively regulates cytokine signaling is still unclear. SOCS2 expression is induced by numerous cytokines and growth factors and it may play a crucial part in the negative regulation of Growth hormone (GH) receptor signal transduction. GH is a PRL-related hormone, which utilizes Growth Hormone Receptor (GHR) which has the typical characteristics of cytokine receptors (Greenhalgh et al. 2005).

SOCS1 inhibits the Jak/Stat pathway by direct binding to the kinase activation loop of Jak, thus inhibiting Stat activation. Mice lacking SOCS1 expression die within three weeks of birth with a low body weight and diseases with a complex pathology such as fatty liver, lymphopenia, the activation of peripheral T-cells and macrophage infiltration of major organs (Elliott and Johnston 2004). Characteristic for the SOCS deficient mouse phenotype was uncontrolled IFN-γ signaling. The mice could be rescued by introducing a second knock-out which deleted either IFN-γ itself or the receptor for IFN-γ (Alexander et al. 1999, Marine et al. 1999).

SOCS3 is structurally similar to SOCS1 but functionally different. SOCS3-mediated inhibition of signaling requires SOCS3 binding to a cytokine receptor. SOCS3 knock-out is embryonically lethal due to uncontrolled Leukemia inhibitory factor (LIF) activation leading to placental deformation (Elliott and Johnston 2004).

The functions and mechanisms of SOCS4-7 are still being studied extensively and future research will reveal the exact roles of these proteins in the regulation of cytokine signaling. For example, SOCS5 appears to be important in

T-cell differentiation signaling and SOCS6 in insulin receptor signaling (Elliott and Johnston 2004).

5.4.4. PIAS proteins

Stat proteins are negatively regulated by Protein inhibitor of activated Stat (PIAS) proteins (Chen et al. 2004). PIAS are a family of constantly expressed Stat-interacting proteins. There are four different PIAS proteins. PIAS1 was found to inhibit Stat1 when a yeast 2-hybrid system was utilized to recognize Stat1-interacting proteins (Liu et al. 1998). The mechanism by which PIAS3 and PIAS1 inhibit Stat activation appears to be a direct protein-protein interaction. PIAS3 was found to bind Stat3 and inhibit its transcriptional activity (Chung et al. 1997). PIAS1 binds to activated STAT1 homodimers and inhibits their binding to DNA, whereas PIAS3 binds to Stat3 homodimers and Stat1/Stat3 heterodimers blocking their DNA binding (Chung et al. 1997, Liu et al. 1998).

PIASx and PIASy do not inhibit Stat activity through direct binding and the mechanims by which they inhibit Stat activation have remained elusive (Chen et al. 2004). However, it is known that all the PIAS proteins have a common domain (RING) which is required for the interaction of small ubiquitin-like protein modifier (SUMO) with target proteins. PIAS proteins can function as SUMO ligases, which means, that they can facilitate the interaction of SUMO with a target protein (Kotaja et al. 2002). The role of this process - termed SUMOylation - in regulation of Stat functions is still somewhat unclear, but recent findings suggest that SUMOylation may have gene-specific effects on Stat-mediated transcription (Ungureanu et al. 2005). It has been suggested that SUMOylation, as ubiquitination, might target Stats outside the nucleus and thus inhibit Stat-mediated transcription. Still, PIASx and PIASy can inhibit the activity of DNA-bound Stats, which suggests additional mechanisms for PIAS-mediated Stat inhibition, probably by chromatin structure regulation (Wormald and Hilton 2004). None of the PIAS family members have been shown to inhibit Stat5a, Stat5b or Stat6 activity.

5.4.5. Protein degradation through the ubiquitin-proteasome pathway

To ensure the degradation of unnecessary proteins, protelytic systems exist in eukaryotic cells. Two important cellular mechanisms have evolved for the degradation of cellular proteins: the vacuolar pathway which contains lysosomes, endosomes and the endoplasmic reticulum, and the cytoplasmic pathway mediated by ubiquitin (Schwartz and Ciechanover 1999). The latter is the most important cytosolic and nuclear proteolytic system, and it functions in two steps. Ubiquitination functions as a "zip code" targeting the unnecessary protein to

degradation. First, the protein directed to degradation is ubiquitinated, in other words, the covalent ligation of ubiquitin, a highly conserved small protein, occurs to several amino acids of the target protein. Second, the polyubiquitinated target protein is recognized by proteasome 26S, a particle with multiple peptidase activity (Ciechanover 1998). The mechanism of ubiquitination requires the functions of three different enzymes termed E1, E2 and E3. E1 is a single enzyme which activates ubiquitin through glycin residue at the C-terminus of ubiquitin. E2 is a group of enzymes and any member of this group can function as ubiquitin-carrier protein. The E1-activated ubiquitin is carried by E2 to a member of the ubiquitin-protein ligase family E3. The E3 group of enzymes then finalizes the ubiquitination by inducing the covalent binding of ubiquitin to a conserved amino acid residue (Lys) on a target protein (Hershko and Ciechanover 1998, Glickman and Ciechanover 2002). Target proteins can be mono- or polyubiquitinated by E ligases. The difference between the effect of mono- or polyubiquitination on a function of a single protein is substantial. Polyubiquitination by at least four ubiquitins to Lys leads to degradation by a proteasome, whereas monoubiquitination does not result in protein degradation but instead it regulates the recycling of membrane proteins and virus budding on the cell surface (Johnson 2004). In the case of IκBα, the E3 ligase covalently ligates ubiquitin preferably after the phosphorylation of target (Chen et al. 1995). The ubiquitin-proteasome pathway regulates the expression and half-lives of numerous proteins that are involved in signal transduction and transcriptional regulation. Many crucial cellular/tissue functions such as apoptosis, development, and immune responses are affected by the ubiquitin-proteasome pathway (Hershko and Ciechanover 1998). In cytokine signaling, the inhibition of the proteasome pathway results in persistent Jak/Stat pathway activation (Callus et al. 1998). However, the mechanisms behind this constant activation are not clear.

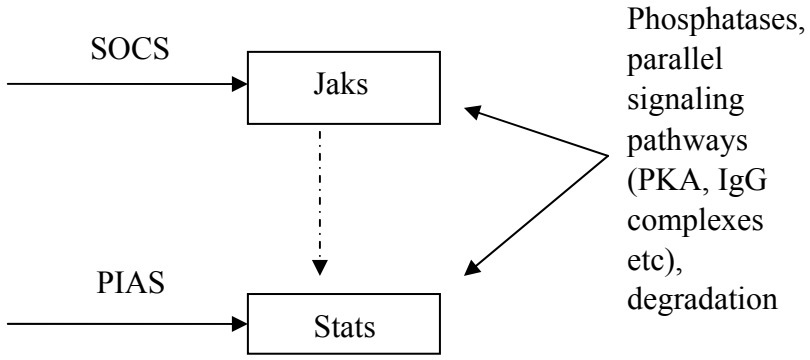

Figure 3. Schematic representation of factors negatively regulating Jak/Stat signaling.

5.5. PKC – a family of kinases with diverse effects on cell functions and signaling

PKC is one of the first protein kinases that was characterized. PKC is a family of serine/threonine kinases, that is, PKC phosphorylates these amino acid residues in the target proteins. Due to the fact that tumor promoting phorbol ester treatment (TPA also known as PMA; phorbol 12-myristate 13-acetate) resulted in prolonged PKC activation (Hecker 1985) an extensive amount of investigation was focused on revealing the role of PKC in signal transduction and cell functions. This was the first time that signal transduction was linked to tumor promotion (Hecker 1985). Since then, our understanding of PKC involvement in numerous cellular functions such as proliferation, apoptosis, differentiation and survival has increased enormously. 12 members of the PKC family have now been characterized. These 12 isoforms are termed with Greek letters.

5.5.1. Structure and classification of PKC isoforms

The PKC family has been classified into three categories: classical, novel and atypical PKCs based on the mechanism of their activation, which is dictated by their structure (Figure 4). All PKC isoforms contain a homologous catalytic C-terminus which consists of two domains C3 and C4. C3 is an ATP binding domain and C4 is a substrate-binding domain (Mellor and Parker 1998). The differences between the isoforms are more evident in the regulatory N-terminus where the domains regulating PKC activity are located.

The classical group of PKCs contains the α, βI, βII (splice variants) and γ isoforms. The N-terminus of these isoforms contains the diacylglycerol (DAG)/phorbol ester binding C1 domain and the Ca^{2+}-binding C2 domain. The C1 domain consists of two repeated cysteine-rich zinc-finger motifs and is found in novel but not in atypical PKCs. The Ca^{2+}-binding C2 domain is specific for classical PKCs even though novel PKCs contain a C2-like domain, which is, however, incapable of binding Ca^{2+}. Novel PKC isoforms are δ, ε, η and θ, and their N-terminus consists of the aforementioned C2-like domain and the C1 domain. The atypical isoforms are ζ, ι and PKMζ (the catalytic fragment of PKC-ζ). They lack the C2 domain, and C1 domain is modified and unable to bind DAG/phorbol ester (Tan and Parker 2003).

These structural domains explain the differences in the activation of PKCs. Classical isoforms containing both functional C1 and C2 domains are activated by DAG/phorbol esters and Ca^{2+}. Novel PKCs are not activated by Ca^{2+} since they contain a non-functional C2 domain. However, they are activated by DAG/phorbol esters since their C1 domain is functional. Atypical PKCs are not activated by Ca^{2+} or DAG/phorbol ester since they do not contain the C2 domain and their C1 domain is not functional (Mellor and Parker 1998).

In addition to these domains, all PKCs have pseudosubstrate sequence (PS) in their regulatory domains. PS bridges the regulatory domain directly to the catalytic domain and blocks the access of substrates into the substrate binding domain at the catalytic domain. This binding is crucial in the PKCs ability to interact with other proteins. It is regulated by PKC-membrane interactions and protein phosphorylation (Liu and Heckman 1998).

5.5.2. Activation of PKCs

The activation of novel PKC isoforms shares common features with both classical and atypical PKC activation. A key component in PKC activation is cellular localization. After translation most PKC isoforms become fully phosphorylated at three well-characterised priming phosphorylation sites and they remain in cytosol in non-activated cells or when no lipid hydrolysis is occurring (Parekh et al. 2000, Tan and Parker 2003). The catalytic domain that contains the substrate-binding pocket is occupied with PS, which ensures that PKC is inactive (Liu and Heckman 1998). Upon cell activation, lipid hydrolysis produces DAG which binds to the regulatory domain of classical and novel PKCs in the presence of phospholipid cofactor (like phospahtidylserine), resulting in conformational change in the PKC molecule. This conformational change results in the removal of PS from its binding site, allowing PKC to actively bind to substrate proteins (Tan and Parker 2003). DAG/phorbol ester binding also increases PKC binding to cellular membranes.

In addition to lipid hydrolysis, protein-protein interactions play a role in PKC activation (Ron and Kazanietz 1999). Several proteins located in different cellular compartments have isoform binding specificity and they appear to target PKCs to the appropriate environment upon PKC activation. Receptors for activated C-kinases (RACKs) bind activated PKCs. RACKs are located on the cell membranes and they bind PKCs isoform-specifically (Schechtman and Mochly-Rosen 2001). Substrates that interact with C kinase (STICKs) are a group of PKC-binding proteins, which also binds to activated PKCs. Importantly, RACKs are not PKC substrates whereas STICKs are (Ron and Kazanietz 1999). Scaffolding proteins such as caveolin and A kinase anchoring protein (AKAPs) bind inactive PKC and appear to be important in regulating the cellular localization of PKC (Ron and Kazanietz 1999).

Lipid-PKC and protein-PKC interactions are by no means the only activating pathways for PKCs. PKCs are also regulated by phosphorylation (Gschwendt 1999). PKC isoforms have conserved serine or threonine residues in the catalytic C-domain of the kinase. These are located in the activation loop (domain C4), turn and hydrophobic motifs. For PKC-δ these sites are threonine 505, serine 643 and serine 663. Threonine 505 regulates kinase stability, whereas serine 643 is an autophosphorylation site and serine 663 a target for an upstream kinase, possibly PKC-ζ (Kikkawa et al. 2002). Tyrosine phosphorylation sites are not conserved among PKC isoforms, and it appears that tyrosine phosphorylation is

isoform-specific modification. PKC-δ is tyrosine phosphorylated on several tyrosine residues. The role of PKC-δ tyrosine phosphorylation on kinase activity and cellular translocation is dependent on the tyrosine residues phosphorylated. ATP, TPA, and H_2O_2 all induce PKC-δ kinase activity, but ATP and TPA do not markedly increase PKC-δ tyrosine phosphorylation, whereas H_2O_2 induced strong tyrosine phosphorylation of PKC-δ (Ohmori et al. 1998). Also, the cellular translocation is markedly different with the aforementioned treatments. Individual tyrosine residues of PKC-δ appear to regulate specific functions of cells as indicated by the increased proliferation of cells expressing PKC-δ with tyrosine 155 to phenylalanine mutation (Kronfeld et al. 2000). Therefore, the role of tyrosine phosphorylation in PKC-δ activation appears to be important, but still poorly understood. This is underscored by the results of Denning et al., showing the negative connection between PKC-δ tyrosine phosphorylation and kinase activity (Denning et al. 1993).

Additional mechanisms for the regulation of PKC-δ activity still exist. All the somewhat complex results mentioned above are related to PKC-δ activation during growth and differentiation. However, PKC-δ also plays a role in apoptosis (Basu 2003). Apoptosis, a cellular suicide, results in cell death through cell size contraction, chromatin condensation and DNA digestion (Kerr et al. 1972). A family of cysteine proteases, caspases, is the main executor of apoptosis. PKC-δ is a substrate for caspases, and PKC-δ activation through caspases occurs through a markedly different method of activation, called degradation (Kikkawa et al. 2002).

Caspases are found as inactive proenzymes in normal cells. It appears that upon activation, two large N-terminal subunits (MW ~20 kDa) dimerize with two small C-terminal subunits (~ 10 kDa) to form a functional protease (Wang and Lenardo 2000). Caspases 8-10 are important in the initiation phase of apoptosis whereas caspase-3, caspase-6, and caspase-7 are important executor-phase proteases of apoptosis. Caspase-2 can function in either phase of apoptosis (Wang and Lenardo 2000). Two major pathways inducing apoptosis exists: extrinsic and intrinsic. Extrinsic apoptosis is associated with the activation of death receptors, whereas intrinsic apoptosis is elicited by cellular stress leading to mitochondria-induced apoptosis (Pinkoski and Green 2005). Tumor-necrosis factor-α (TNF-α) induces cell death through its receptor activation, converging to initiator caspases such as caspase-8 (Ashkenazi and Dixit 1998). Cytochrome c release from mitochondria induces apoptosis through the activation of initiator caspases for example caspase-9 (Green and Reed 1998). PKC-δ is activated through degradation by executor caspase-3. Caspase-3 cleaves PKC-δ at the $DMNQD^{330}N$ site at the hinge region between catalytic and regulatory domains resulting in the release of a 40 kDa catalytic fragment. This fragment then translocates into cell compartments, for example, the nucleus, mitochondria or the plasma membrane on the basis of apoptotic stimuli (TPA, UV or UVB radiation, cisplatin) and the cell type (Basu 2003).

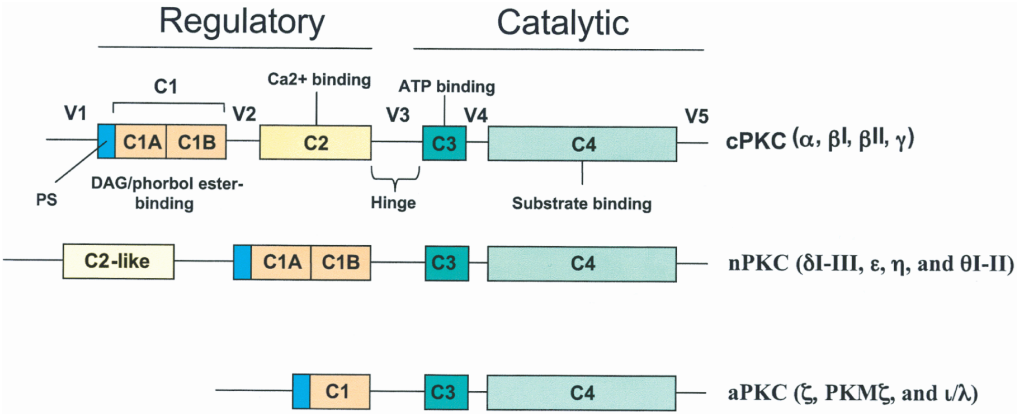

Figure 4. Structure of PKCs. A schematic representation of different PKC isoforms and their structure. Reproduced with permission from Tan and Parker (2003, *Biochem. J.* 376 pp. 547). © The Biochemical Society.

5.5.3 Immunological functions of PKC

Despite fairly broad substrate specificity *in vivo*, different PKC isoforms possess important cellular and immunological functions as demonstrated by mice lacking the expression of different PKC isoforms (Tan and Parker 2003). The viability of mice lacking the functional expression of a member of the PKC family suggests that redundancy between PKC isoforms exists. However, some PKC isoforms have distinguishable immunological and physiological functions. PKC-β knock-out mice have impaired B-cell receptor signaling which leads to decreased humoral responses of adaptive immunity (Leitges et al. 1996). In addition, mast cells from these animals are defective in degranulation and IL-6 production (Nechushtan et al. 2000). PKC-δ deficiency results in decreased B-cell anergy, hyperproliferative B-cells and the formation of autoreactive antibodies as well as enhanced degranulation from mast cells (Leitges et al. 2002, Mecklenbrauker et al. 2002, Miyamoto et al. 2002). It is of interest that smooth muscle cells (SMC) apoptosis in PKC-$\delta^{-/-}$ mice was dysregulated, indicating a more general role for PKC-δ in the regulation of apoptosis and proliferation (Leitges et al. 2001). Macrophages from mice without the functional expression of PKC-ε were hyporesponsive to IFN-γ and LPS due to defects in nuclear factor κB (NFκB) nuclear translocation, and these mice were unable to mount a functional immune response against gram-positive or gram-negative bacteria (Castrillo et al. 2001). The lack of PKC-θ expression results in defective T-cell receptor (TCR) signaling (Sun et al. 2000). PKC-ζ regulates B-cell signaling, and the loss of this isoform results in the inhibition of B-cell growth and survival (Martin et al. 2002). Despite the fact that knock-out models provide interesting and detailed information of the possible roles of different PKCs in immune functions, one has

to keep in mind several restrictions of these models. Firstly, the effect brought about by the loss of PKC expression may already affect at the developmental level and the phenotype is not truly mirroring the defect in an adult animal. Secondly, defects in PKC upstream signaling which might explain changes in PKC downstream signaling have not been addressed in these studies (Tan and Parker 2003). The question of the possible compensatory mechanisms is also an important one in studies conducted with knock-out animals. The specific deletion of a certain gene may result in the activation of unexpected adaptive compensatory mechanisms (Pich and Epping-Jordan 1998).

PKC-isoform	PHENOTYPE OF NULLIZYGOUS MICE
PKC-β	Impaired B-cell survival and BCR signaling, defects in mast cell functions
PKC-δ	Hyperproliferative B- and mast cells, impaired B-cell anergy
PKC-ε	Impaired macrophage activation
PKC-θ	Impaired TCR signaling
PKC-ζ	Impaired BCR signaling

Table 2. Immunological phenotypes of PKC-deficient mice. Modified from Tan and Parker 2003.

6. AIMS OF THE STUDY

During the past two decades, the understanding of cytokines regulating myeloid cell growth and differentiation has increased significantly. The signaling events triggered by cytokines have been elucidated in detail and the mechanisms by which hematopoietic cytokines induce signaling are fairly well characterized. Signaling via the Jak/Stat pathway is a central mechanism for cytokine signaling. However, mechanisms of the negative regulation of the Jak/Stat pathway remain only partially understood.

The detailed aims of this study were:

1. To study the regulation of IL-3-induced and IFN-γ-induced Jak2 activation during macrophage differentiation and activation.

2. To characterize the molecular mechanisms of Jak regulation.

3. To elucidate the role of PKC isoforms in macrophage differentiation signaling.

7. MATERIALS AND METHODS

7.1. Cell culture and transfections

If not otherwise stated, all cell lines were obtained from American Type Culture Collection (Manassas, VA). 293, Cos7, Wehi and primary mouse fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen) and antibiotics (100 U of penicillin/ml and 100 μg of streptomycin/ml). FDCP-1, 32D, HL-60, THP-1 and primary human monocytes were grown in RPMI medium (Invitrogen) containing 10 % FBS and antibiotics (100 U of penicillin/ml and 100 μg of streptomycin/ml). BaF3 cells were a kind gift from Dr M. Heim. 32D-PKC-α, -δ and -ε expressing cells were provided by Dr W. Li (Mischak et al. 1993). FDCP-1 cells expressing mutated c-Fms (M-CSF receptor) were from Dr. R.P. Bourette (Bourette et al. 1995). IL-3-dependent cell lines FDCP-1, BaF3 and 32D cells were supplemented with 5 % Wehi cell supernatant for IL-3. 293 and Cos7, transfections were made by electroporation with a Bio-Rad gene pulser at 220 V/960μF and 260 V/960μF respectively.

Stable cell lines that expressed conditionally PKC-δ wild-type or kinase-negative were established by electroporation of 10 cm dish of subconfluent 293 cells with pIND expression vector (Invitrogen) containing PKC-δ constructs in EcoRI site (PKC-δ wild-type) or EcoRI-XbaI site (PKC-δ kinase negative) together with pVgRXR plasmid (Invitrogen) containing the modified heterodimeric ecdysone/retinoid X receptor. 48 hours after transfection, selection antibiotics (200 μg/ml of Zeocin and 800 μg/ml of G418) were added to the cells. After two weeks, the viable cell colonies were picked up. The expression of PKC-δ of different clones was analysed from total cell lysates by Western blotting with PKC-δ antibody (Gibco BRL Life Technologies, Gaithesburg MD) after PKC-δ expression was induced by Muristerone (Invitrogen), an analog of ecdysone (5 μM, 20 hours). 32D and 32D-PKC-δ cells expressing Jak2 were established by transfecting 10 million 32D or 32D-PKC-δ cells with 5 μg of Jak2 in SalI site of pBabe-Puro expression vector by electroporation 240V/960μF. 24 hours after electroporation, selection antibiotic (2,5 μg/ml puromycin) was added to the cells and after a fortnight, the viable cells were dilution-cloned. The expression of Jak2 protein in different clones was investigated by Western blotting with antibody against Jak2 (kind gift from Dr. J. Ihle).

7.2. DNA constructs

Expression vectors for HA-tagged Jak2 WT and KN (Saharinen et al. 1997), Stat1 (Schindler et al. 1992) and Stat5a (Wakao et al. 1994) were obtained by adding HA-epitope to the 3´end of the respective kinase expressed in pCIneo expression vector. The Y1007 mutation of Jak2 was obtained by direct PCR mutagenesis to Jak2 WT with following primers; 5' TGCCGCAGGACAAAGAATTCTACAAAGTAAAGGAGCCA and 3'TGGCTCCTTTACTTTGTAGAATTCTTTGTCCTGCTGCGGGA. HA-tagged ubiquitin and His_6-ubiquitin were kindly provided by Dr. D. Bohman. SOCS-1, SOCS-1ΔSB and SOCS-3 expression vectors were provided by Dr D. J. Hilton (Hilton et al. 1998, Zhang et al. 1999). PKC-δ WT and KN expression vectors were obtained from Dr. W. Li (Li et al. 1995).

7.3. Antibodies and cytokines

Antibodies against PKC-α, -β, -δ (C and N-terminus), -ε and -ζ isoforms were purchased from Gibco BRL (Gaithesburg, MD), Chemicon International Inc (Temecula, CA) or Transduction Laboratories (Lexington, KY). Antibodies against Stat-1 (C-terminus) and IL-3R β-chain were purchased from Transduction Laboratories. Anti-influenza virus haemagglutinin (HA) epitope antibody (clone 12CA5) was purchased from Berkeley Antibody Company (Richmond, CA). Anti-phosphotyrosine-antibody (clone 4G10) was from Upstate Biotechnology, (Lake Placid, NY) and anti-PStat1 from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-Fms-antibody was targeted against the cytoplasmic domain of Fms and was provided by Dr. R.P. Bourette (Bourette et al. 1997). Polyclonal Jak2 antibody was a kind gift from Dr J. Ihle. Antiubiquitin antibody mAb-Ubi-1 was from Sigma-Aldrich, and anti-SOCS-1 and anti-SOCS-3 monoclonal antibodies were provided by Dr D. J. Hilton (Zhang et al. 1999). Murine IL-3 was purchased from PeproTech (London, Great Britain), murine IFN-γ from Diaclone (Besancon Cedex, France), human IFN-γ from Immugenex Corp (Los Angeles, CA) and murine M-CSF from R&D Systems.

7.4. Immunoprecipitation and Western blotting

After indicated stimulations, cells were lysed in Triton lysis buffer (50 mM Tris-HCl, pH 7.5, 10% glycerol, 150 mM NaCl, 1 mM EDTA, 1% Tritonx100,

50 mM NaF, and 1 mM Na_3VO_4) supplemented with protease inhibitors. For coimmunoprecipitation studies, cells were lysed in 1% NP40 lysis buffer (as Triton lysis buffer but with Nonidet P40 as detergent). Immunoprecipitations were carried out from equal protein amounts as previously described (Saharinen et al. 1997). The protein concentrations were determined using the BioRad Protein Assay System (Bio-Rad Laboratories, Hercules, CA). Immunoprecipitates were separated by SDS-Polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane (Micron Separation Inc, Westborough MA). Immunodetection was performed by using specific primary antibodies, biotinylated anti-mouse or anti-rabbit secondary antibodies (Dako A/S, Denmark), and streptavidin-biotin horseradish peroxidase-conjugate. Immunodetection was performed with the ECL system (Amersham Pharmacia Biotech, Buckinghamshire, UK).

7.5. Electrophoretic mobility shift assay (EMSA)

Cells were lysed in WCE lysis buffer (50 mM Tris-HCL, pH 8, 0,5% NP-40, 10% glycerol, 0,1 mM EDTA, 150 mM NaCl, 50 mM NaF, 0,5 mM Na_3VO_4) supplemented with protease inhibitors for electrophoretic gel-mobility shift assay. Annealed GAS oligonucleotide from the murine IRF-1 gene 5'-CTAGAGCCTGATTTCCCCGAAATGATGAG-3' was end-labeled by T4 polynucleotide kinase using [γ-^{32}P] ATP. Cell lysates (10 μg), poly-dI-dC (240 ng/μg/ml, bovine serum albumin (BSA;1,5 mg/ml) and ^{32}P-labeled IRF oligonucleotide (0,5ng) were incubated for 30 minutes at room temperature. The reactions were then separated in 4.5% TBE-PAGE (2.2 × TBE) followed by autoradiography.

7.6. Flow cytometry

After treating human peripheral blood monocytes with IFN-γ and/or TPA for 20 hours, the cells were first washed twice with PBS and then suspended in PBS. The cells were stained with FITC-conjugated anti-HLA-DR antibody (BD Biosciences, San Jose, CA) or with control antibody, isotype-matched FITC - conjugated anti-IgK2aκ (BD Biosciences) for 45 minutes at 4°C and washed twice with PBS. The cells were analysed with FACScalibur (BD Biosciences) and the expression was analysed from the gated monocyte population.

7.7. Protein kinase C and Jak2 *in vitro* kinase activity measurements and Jak2 phospho-amino acid analysis

For PKC-δ kinase assay, either anti-HA immunoprecipitation or PKC immunoprecipitation was performed as indicated. Immunocomplexes were then suspended in kinase reaction buffer (0.04 mg/mL phosphatidyl-L-serine, 10% glycerol, 0.1 mM $CaCl_2$, 0.02% Triton-X-100, 10 mM $MgCl_2$, 20 mM HEPES, pH7.4) in the presence of [γ^{32}P] ATP (0.25 μCi/mL), and the reaction was allowed to proceed for 30 minutes at 30°C. The proteins were resolved in SDS-PAGE, and the gel was dried in a vacuum (2 hours 80 °C) and exposed for autoradiography.

Jak2 substrate kinase assay was performed from immunoprecipitated proteins by suspending the immunoprecipitates in kinase assay buffer (10 mM HEPES pH 7.4, 50 mM NaCl, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 50 mM NaF, 0,1 mM Na_3VO_4) containing 1 mM DTT, synthetic peptide corresponding to the tyrosine phosphorylation site of Stat1 (GPKGTGYIKTELISVS) and 10 μCi [γ^{32}P]-ATP. The reactions were allowed to proceed for 10 minutes in room temperature, followed by boiling in Laemmli sample buffer and separation in 20% SDS-PAGE and autoradiography.

For Jak2 phospho-amino acid analysis, Jak2 and PKC-δ were transfected to Cos7 cells. After two days, cells were starved overnight and labeled with ^{32}P-orthophosphate (Amersham) in a phosphate-free medium (Sigma) for 3 hours. The cells were then lysed in Triton lysis buffer, and Jak2 was immunoprecipitated. Immunocomplexes were separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham). Proteins were visualized by autoradiography, and the band corresponding to Jak2 was excised for further analysis. For the phospho-amino acid analysis, proteins were hydrolyzed in 6 M HCl for 2 hours at 110°C. Lysates were lyophilized and resolved in pH 1.9 buffer containing standard phospho-amino acids (o-Phospho-DL-serine, o-Phospho-DL-threonine, o-Phospho-DL-tyrosine; Sigma). Phospho-amino acids were separated by 2-dimensional electrophoresis. Standard amino acids were visualized by ninhydrin staining, and autoradiography was used to detect phosphorylated amino acids

7.8. Thymidine incorporation and apoptosis assays

Growing 32D, 32D-PKC-δ and 32D-PKC-δ-Jak2 cells were stimulated with IL-3, TPA and AG490 (Jak inhibitor). ^{3}H-thymidine (1μCi/well) was added 18 hours later to the cells for 6 hours followed by harvesting and analysing the DNA-incorporated thymidine. Cells were then washed three times with PBS and cell-associated radioactivity was determined by liquid scintillation counting.

Apoptosis was determined by analysing the DNA fragmentation of chromosomal DNA (Ueno et al. 1998) on 2 % agarose gel. Alternatively, In Situ Cell Death Detection Kit (TUNEL staining) was used according to the manufacturer's instructions (Boehringer Mannheim, Germany).

7.9. Subcellular fractionation

Stimulated cells were lysed on ice for 10 min in 600 µl hypotonic buffer (10 mM HEPES, 1 mM $MgCl_2$, 1 mM EDTA, 4 mM di-isopropylfluorophosphate, 0.5% aprotinin, 10 µg/ml leupeptin, pH 7.5). The particulate fraction and the soluble fraction were separated with a 30 min centrifugation (4°C, 100 000 *g*). The soluble fraction was obtained by centrifuging the supernatant once again (30 min, 4°C, 100 000 *g*). To obtain the particulate fraction and to eliminate soluble contaminations, the pellet was washed in 1 ml hypotonic buffer and then lysed in 600 µl radio immunoprecipitation assay (RIPA) lysis buffer (1% deoxycholic acid, 1% Nonidet P-40 (NP-40), 0.1% sodium dodecyl sulfate (SDS), 10 mM Tris-base, 150 mM NaCl, 0.5% aprotinin, pH 7.4). To purify the particulate fraction, insoluble particles were isolated with a 30 min centrifugation (4°C, 100 000 *g*) and solubilized to Triton-X lysis buffer (described in chapter 7.4).

7.10. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA isolation from stimulated 32D cells was done with the Trizol reagent (Gibco-BRL Life Technologies). The obtained total RNA was used for the first-strand cDNA synthesis with monkey murine leukemia virus transcriptase (Gibco-BRL Life Technologies) and random hexamers (Amersham Pharmacia Biotech), according to the manufacturers' protocol. PCR was performed with primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using the GATGACATCAAGAAGGTGGTG primer for sense and the GCTGTAGCCAAATTCGTTGTC primer for antisense. For Pkare, the PCR primers were ACTGGGACCTTTGGCCGTGT sense and CTATCCTCCAGTTTCTTGGC antisense. PCR conditions were: 94°C, 1 min; 55°C, 1 min; 72°C, 3 min. The reaction was repeated 25 times, and 25 µl of the product was loaded on a 1.2% agarose gel containing ethidiumbromide. For FDC-P1 myeloid precursor cells expressing Fms wt (FD-Fms-wt) cells, RNA was extracted from cells stimulated with IL-3 or M-CSF for 1, 2, or 3 days, using the Rneasy kit (Qiagen, Valencia, CA). Total RNA (2 µg) was reverse-transcribed using random hexamers and the Omniscript RT kit (Qiagen). One-tenth of the first-strand cDNA synthesis reaction was used as template for the PCR reactions using the Pkare and GAPDH primers (described above). PCR

conditions were: 95°C, 30 s; 60°C, 30 s; 72°C, 1 min. The reaction was repeated 35 times and 25 µl of the product was loaded on a 1.5% agarose gel containing ethidiumbromide.

8. RESULTS

8.1. PKC-δ inhibits IL-3 and IFN-γ induced Jak2 kinase activity (I, II)

Macrophages differentiate from myeloid progenitor cells which, are located in bone marrow. During the differentiation the cells also move from the bone marrow into tissues. Therefore, studying macrophage differentiation is challenging, and several cell-line models have been developed to assist study of differentiation. One such model is 32D-cells, IL-3 dependent murine myeloid progenitors. In these cells, ectopic PKC-α and -δ expression induces macrophage differentiation (Mischak et al. 1993). To investigate the effect of PKC-induced differentiation on IL-3-induced signaling, 32D cells either wild type or expressing the PKC-α, -δ or -ε isoform were starved overnight and treated various times with TPA followed by a 15 min stimulation with IL-3. Total cellular phosphorylation was clearly reduced in cells expressing PKC-α or -δ. Especially, the phosphorylation of 120-140 kDa proteins was reduced, which suggests that IL-3 receptor β chain (IL-3Rβ) or Jak2 might be regulated by PKC-α or -δ. Since the catalytic domain of PKC-δ mediates macrophage differentiation (Wang et al. 1997), we concentrated in our studies to this isoform, as we studied PKC-mediated signaling during TPA-induced macrophage differentiation. Further studies of IL-3R β-chain and Jak2 revealed that IL-3R β-chain tyrosine phosphorylation is inhibited due to decreased Jak2 tyrosine phosphorylation. The PKC-δ-mediated inhibition of IL-3 signaling might be due to IL-3R β-chain internalisation and degradation, or Jak2 degradation. However, the protein levels of IL-3R β-chain and Jak2 remained unaltered which indicates that receptor uptake, protein degradation or translocation did not play a role in this rapid down-regulation of IL-3 signaling. In other cell systems, such as IL-3 dependent pro-B cell line Ba/F3, ectopic expression of PKC was not required for IL-3-induced Jak2 inhibition. In Ba/F3 cells TPA mainly induced the activity of the PKC-α isoform (data not shown).

In addition to the activation of the Jak2 kinase, the binding of IL-3 to its cognate receptor activates parallel signaling pathways including Shc/Ras/MAPK leading to *c-fos* expression (Gotoh et al. 1996). The activation of ectopically expressed PKC-δ did not influence the IL-3-induced phosphorylation of this pathway as detected by the prominent IL-3-induced tyrosine phosphorylation of the Shc adaptor protein in both 32D and 32D-PKC-δ cells (data not shown).

The phosphorylation of tyrosine residues in the kinase activation loop of Jak2 (A-loop) through auto- or transphosphorylation by another Jak kinase induces Jak2 kinase activity (Feng et al. 1997). The effect of PKC-δ-mediated decrease in the tyrosine phosphorylation of Jak2 to Jak2 kinase activity was confirmed next. Jak2 and PKC-δ were co-expressed in COS7 cells and immunoprecipitated, and Jak2 was subjected to *in vitro* kinase assay. As expected, Jak2 demonstrated a clearly reduced ability to phosphorylate a substrate peptide (Stat1 tyrosine phosphorylation site-peptide). When COS7 cells were co-transfected with the atypical PKC-ζ-isoform, which has no effect on Jak2 tyrosine phosphorylation, the kinase activity of Jak2 was not influenced.

The effect of PKC activity on cytokine-induced Jak2 activity in different cytokine receptor systems was studied to better understand whether PKC-mediated Jak2 inhibition is a more commonly utilized system for the negative regulation of cytokine signaling. IFN-γ stimulation induces the rapid tyrosine phosphorylation of Jak2 and Stat1 (Bach et al. 1997). Myelomonocytic human HL-60 and THP-1 cells were starved overnight, followed by stimulation with TPA and IFN-γ. As expected, IFN-γ induced the strong phosphorylation of Jak2 and Stat1. However, if the cells were pretreated for 30 minutes with a potent PKC activator TPA before IFN-γ stimulation, the tyrosine phosphorylation of both Stat1 and Jak2 was clearly reduced. To identify the activated PKC isoforms, *in vitro* kinase assays were performed on TPA-treated HL-60 and THP-1 cells. The kinase activity of the classical PKC-α and novel PKC-δ isoforms were clearly increased as indicated by the increased autokinase activity. Atypical PKC-ζ activity was not altered by the TPA treatment.

Both IL-3 and GM-CSF receptors signal via the common β-chain and therefore intracellular signaling elicited by these receptors has numerous common features (Barreda et al. 2004). The effect of PKC activation on GM-CSF-induced Jak2 activity was investigated. Surprisingly, GM-CSF-induced Jak2 tyrosine phosphorylation was not decreased upon TPA-mediated PKC activation (data not shown).

8.2. Inhibition of Jak2 by PKC-δ requires PKC-δ kinase activity and involves serine/threonine phosphorylation of Jak2 kinase (I, II)

There are several mechanisms by which PKC-δ might regulate cytokine-induced Jak2 tyrosine phosphorylation. Either SOCS proteins or protein phosphatases (Greenhalgh and Hilton 2001) might be activated by PKC-δ. In all studied cell lines, the pretreatment of cells with phosphatase inhibitor (Na_3VO_4) had no effect on TPA-induced inhibition of either IL-3 or IFN-γ-induced Jak2 activation (data not shown). Inhibition of Jaks by the SOCS protein requires *de novo* synthesis of SOCS proteins. TPA treatment for 45 minutes up-regulates SOCS3 expression in mouse fibroblasts (Terstegen et al. 2000). TPA treatment of HL-60

or THP-1 cells for 30 minutes had no effect on SOCS-3 protein levels as determined by Western blotting (data not shown). Since PKC is a family of serine/threonine kinases, the inhibition of Jak2 might also occur via the direct phosphorylation of these amino acid residues of Jak2 by PKC-δ. To investigate the latter, we utilized the previously described mutant of PKC-δ (Li et al. 1995). The mutation of lysine 376 to arginine at a PKC-δ catalytic domain inhibits kinase activity of PKC-δ (PKC-δ-KN). As described above, when ectopically expressed in 32D cells TPA induces macrophage differentiation in PKC-δ wild type (PKC-δ-WT) cells. However, in 32D cells expressing PKC-δ-KN (32D-PKC-δ-KN) TPA was unable to induce macrophage differentiation (Li et al. 1995).

The role of PKC-δ kinase activity on IL-3-induced Jak2 activity was studied first. Previously described 32D cell lines with a constant ectopic expression of both PKC-δ-WT and PKC-δ-KN were used. PKC-δ-KN was unable to inhibit IL-3-induced Jak2, suggesting a role for the serine/threonine kinase activity of PKC-δ.

To verify the results in another receptor setting, IFN-γ-induced Jak2 was used as a target for PKC-δ-WT and PKC-δ-KN. To avoid the constant ectopic expression of PKC-δ, an important regulator of crucial cellular functions (Gschwendt 1999), an inducible system for PKC-δ expression was constructed as described in detail in chapter 7. Briefly, human embryonic kidney cells (HEK293) were co-transfected with expression vectors for PKC-δ (WT and KN respectively) and modified insect steroid receptor containing the mammalian VP16 domain. The induction of the expression was obtained by adding ligand (Muristerone) to the insect steroid receptor which induced transcription through the VP16 domain. Importantly, the inducible expression of PKC-δ-WT inhibited the IFN-γ-induced tyrosine phosphorylation of Jak2 as expected, whereas PKC-δ-KN failed to inhibit the IFN-γ-induced tyrosine phosphorylation of endogenous Jak2 in these cells.

To confirm the serine/threonine phosphorylation of Jak2 by PKC-δ, the two kinases were cotransfected in COS7 cells. Jak2 was immunoprecipitated and subjected to *in vitro* PKC kinase assay where PKC transfers ^{32}P labelled γATP to amino acid residues of PKC targets. Thus, the co-precipitating PKC-δ was allowed to phosphorylate Jak2. Followed by separation on SDS-PAGE and autoradiography, the band corresponding to Jak2 was excised from the gel and subjected to digestion with trypsin, 2-dimensional gel electrophoresis and phospho-amino acid analysis revealing the phosphorylation of both the serine and threonine amino acid residues of Jak2. The minimal domain of Jak2 phosphorylated by PKC-δ was determined in co-transfection assays. PKC-δ expression vector was co-transfected with different Jak2 domains in COS7 cells, and the Jak2 constructs were immunoprecipitated and subjected to *in vitro* PKC kinase assay. The kinase domain of Jak2 was the minimal requirement for phosphorylation by PKC-δ (data not shown).

As Jak2 tyrosine phosphorylation by PKC-δ is a critical step in IL-3 and IFN-γ-induced signal transduction, the role of Jak2 tyrosine phosphorylation on

interaction with PKC-δ was studied. Interestingly, the functional interaction of Jak2 and PKC-δ also required Jak2 kinase activity, since the co expression of PKC-δ with kinase negative Jak2 (Jak2-KN) did not induce the phosphorylation of Jak2. It could be envisaged that Jak2-KN might be localised differentially from wild type Jak2 (Jak2-WT). However, all experiments performed with Jak2-WT or Jak2-KN transfections revealed equal Jak2 protein levels, suggesting equal cellular distribution of Jak2-WT and Jak2-KN.

8.3. M-CSF activates the δ isoform of PKC family and induces the expression of Pkare (III)

M-CSF regulates the differentiation and growth of macrophage precursor cells and it induces the activation of PKC (Imamura et al. 1990). However, the isoform specificity and molecular mechanisms of M-CSF-induced PKC activation have remained elusive. PKC isoforms have various, even opposing functions, as demonstrated by the phenotypes of mice genetically modified to lack the expression of a single PKC isoform (Tan and Parker 2003). To characterize the PKC isoform regulated by M-CSF, we studied the effect in a well-characterized model of M-CSF-induced differentiation, namely murine FDCP1 cells. These cells are unresponsive to M-CSF, but the ectopic expression of M-CSF receptor (Fms) renders them responsive to M-CSF and induces macrophage differentiation in these cells (Bourette et al. 1995). The signaling events elicited by M-CSF receptor activation are rapid and occur within seconds and minutes (Bourette et al. 1997). To study PKC activation, FDCP1 cells expressing M-CSF receptor were starved overnight and then stimulated briefly (45 s) with M-CSF. The cells were lysed and different PKC isoforms were immunoprecipitated and subjected to PKC *in vitro* kinase assay. M-CSF induced the rapid and transient activation of novel PKC-δ isoform, whereas the classical α- and β-isoforms, the novel ε-isoform and the atypical ζ-isoform were not rapidly affected by Fms activation. M-CSF-induced PKC-δ activation was measured by the catalytic fragmentation, tyrosine phosphorylation and cellular translocation of PKC-δ. The tyrosine phosphorylation kinetics of PKC-δ were rapidly declining already after 5 minutes of M-CSF treatment. Similarly, membrane translocation was rapid and transient. Other tested hematopoietic growth factors such as GM-CSF and IL-3 did not have an effect on PKC-δ tyrosine phosphorylation.

To study the molecular mechanisms regulating the M-CSF-induced activation of PKC-δ, FDCP1 cells expressing M-CSF receptor mutants were used. Tyrosines 721 and 807 of the M-CSF receptor are important amino acid residues in regulating intracellular signaling leading to M-CSF-mediated growth and differentiation (Hamilton 1997). Interestingly, the mutation of tyrosine 807 to phenylalanine abrogates M-CSF-induced differentiation totally whereas the mutation of tyrosine 721 only reduces differentiation but does not inhibit it

totally (Bourette et al. 1995). Tyrosine 721 regulates PI3 kinase binding to the receptor and the subsequent PLC-γ2 binding and activation whereas tyrosine 807 regulates PLC-γ2 binding and activation (Bourette et al. 1997). This suggests an indispensable role for signaling pathways regulated by tyrosine 807 in M-CSF-induced differentiation. Since PLC-γ2 is activated by tyrosine 807 of M-CSFR, it could play a key role in the regulation of M-CSF-induced differentiation (Bourette et al. 1997). Interestingly, PLC-γ also plays an important role in the activation of the classical and novel PKC isoforms, since PLC can hydrolyse both phosphatidylinositol (PI) and phosphatidylcholine (PC) to yield diacylglycerol DAG, which is an important activator of these PKC isoforms (Newton 1997). Tyrosine 807 mutation in FDCP1 cells resulted in the dramatic reduction of PKC-δ tyrosine phosphorylation and membrane translocation.

A novel protein kinase (PRKX) was described to be an important regulator of macrophage differentiation in human macrophages as well as in promyelocytic HL-60 cells (Semizarov et al. 1998). The mouse homologue of PRKX, Pkare, has been associated with neuronal differentiation (Blaschke et al. 2000). However, the exact role of Pkare functions is still elusive. To study the possible expression of Pkare in the terminal differentiation of macrophages, FDCP1-Fms-WT and 32D-PKC-δ cells were induced to differentiate. TPA was used, for the differentiation of 32D-PKC-δ cells and M-CSF was utilized for FDCP1-Fms-WT. Total RNA from differentiated cells was extracted, reverse transcribed to cDNA and basic PCR was performed. Pkare expression increased during both M-CSF-induced differentiation in FDCP1 cells, and TPA induced differentiation in 32D-PKC-δ cells. This would imply that Pkare is a macrophage-differentiation-associated gene, and Pkare expression is regulated by an unknown mechanism through PKC-δ. The kinetics of Pkare expression were markedly different between TPA induction in 32D-PKC-δ cells and M-CSF induction in FDCP1-Fms-WT cells.

8.4. Cytokine-induced Jak2 is negatively regulated by the Ubiquitin-proteasome pathway (IV)

Protein degradation is a vital mechanism in sustaining homeostasis in the body. Extracellular proteins are degraded via pinocytosis or receptor-mediated uptake and nonspecifically degraded in lysosomes, i.e. protein taken up by pinocytosis is automatically degraded (Glickman and Ciechanover 2002). Inside the cell, the rapid protein turnover of intracellular proteins is critical for sustaining intracellular homeostasis. The cytosolic pathway for protein degradation occurs through proteasomes. A proteasome is a large multicatalytic protease responsible for selective intracellular protein degradation. Targeting to proteasomes occurs via ubitiquitination, the covalent ligation of ubiquitin, a highly conserved small protein, to several amino acids of target protein (Ciechanover 1998, Ciechanover et al. 2000). The inhibition of proteasomes by pharmacological substances,

proteasome inhibitors, results in prolonged cytokine signaling through the IL-3 receptor (Callus and Mathey-Prevot 1998).

The scope of this study was to investigate whether IL-3 or IFN-γ-induced Jak2 might be negatively regulated by targeting it to the proteasomes through ubiquitination. 32D and COS7 cells were starved overnight and treated with proteasome inhibitors followed by cytokine stimulation (IL-3 for 32D cells and IFN-γ for COS7 cells). Interestingly, Jak2 appears to be monoubiquitinated in the resting cells, but cytokine stimulation induces the polyubiquitination of Jak2 as indicated by the characteristic ubiquitination smear in Western blots. This would imply different functions for mono- and polyubiquitination as has been described (Johnson 2002). Ubiquitination was stabilized upon the inhibition of proteasomes by inhibitors. The mechanism of ubiquitination of Jak2 required the tyrosine phosphorylation of Jak2 since Jak2-WT was highly ubiquitinated whereas neither Jak2-KN (K882E substitution) nor Jak2-YF (Y1007F substitution) were ubiquitinated in co expression studies with Jak2-WT, Jak2-KN or Jak2YF with ubiquitin.

PKC-δ has been suggested to be involved in the serine phosphorylation and ubiquitination/degradation of Insulin receptor substrate-1 (IRS-1) (del Rincon et al. 2004). This raised the possibility that PKC-δ might additionally regulate Jak2 through ubiquitination. However, co-transfection experiments with PKC-δ and Jak2 did not result in the enhanced ubiquitination of Jak2 whereas Jak2 tyrosine phosphorylation was clearly reduced (data not shown). This suggests distinct roles for PKC-δ induced serine/threonine phosphorylation and ubiquitination in the regulation of Jak2 activity.

8.5. FcγR activation inhibits IFN-γ induced Stat1 activation and increases PKC-δ activation (II)

Immunocomplexes are potent regulators of macrophages and they also regulate the signaling of cytokines, such as IFN-γ and IL-10 (Feldman et al. 1995, Ji et al. 2003). IFN-γ-induced Stat1 activation has been shown to be negatively regulated by immunocomplexes (Feldman et al. 1995). However, opposing results have also been observed (Barrionuevo et al. 2003).

The effect of immune complexes on IFN-γ signaling was studied to better understand the mechanisms underlying differentially induced macrophage activation. The cross-linking of FcγRs activates these receptors and induces downstream signaling through ITAM motifs (Ravetch and Bolland 2001). FcγR cross-linking was obtained through the antibody-mediated cross-linking of the Fab (antibody binding domain) domains of IgG. FcγR activation resulted in the rapid inhibition of IFN-γ-induced Stat1 tyrosine phosphorylation in promyelocytic HL-60 cells. Interestingly, we did not detect FcγR-mediated inhibition of IFN-γ-induced Stat1 unless the HL-60 cells were activated with IFN-γ (50 ng/ml) for 24 hours before the cross-linking (data not shown).

FcγR-induced intracellular killing of *Staphylococcus aureus* involves the activation of certain PKC isoforms (Zheng et al. 1995). FcγR also increases PKC-δ, -ε and -ζ activity in IFN-γ treated U937 cells (Melendez et al. 1999). These findings combined with our results demonstrating that FcγR cross-linking also induced the tyrosine phosphorylation of several cellular proteins, including 70-80 kDa proteins, led us to study more closely the possibility that PKC-δ might be activated and regulating IFN-γ signaling in our experimental setting.

Translocation to cellular membranes is a hallmark of PKC-δ activation (Ohno et al. 1994). In IFN-γ-primed HL-60 cells, the cross-linking of FcγR resulted in the rapid and transient membrane translocation of PKC-δ. In addition, the tyrosine phosphorylation of PKC-δ was slightly increased by FcγR activation (data not shown). There are at least six non-conserved tyrosine residues in PKC-δ (Y52, Y155, Y187, Y311, Y332 and Y565) and one conserved among the PKC isoforms (Y512) which can be phosphorylated. The phosphorylation of these residues appears to be specific to the stimulus and cell type (Kronfeld et al. 2000, Kikkawa et al. 2002), but the residues phosphorylated after FcγR activation were not determined in this study.

8.6. PKC activation inhibits IFN-γ-induced responses in human monocytes (II)

IFN-γ-induced signaling leads to Jak1/Jak2 activation and subsequently Stat1 activation (Pestka et al. 2004). Upon activation, Stat1 homodimers bind to specific sequences of DNA (GAS) to regulate the expression of probably hundreds of genes (Ramana et al. 2002, Paukku and Silvennoinen 2004). To study the effect of PKC activation on IFN-γ responses, human monocytes were purified from peripheral blood. 30 minutes of TPA treatment of the human monocytes before IFN-γ stimulation decreased Stat1 DNA binding clearly, as determined by EMSA. The target DNA for Stat binding was the GAS site from murine interferon regulatory factor 1 (IRF-1) gene.

An important gene up-regulated by IFN-γ in monocytes is Major histocompatibility complex class II (MHCII, also known as Human leukocyte antigen II, HLAII). MHCII is a key molecule in presenting foreign material to CD4-positive T-cells. MHCII molecules contain α-chain and β-chain, and they can be constructed from three subtypes: HLA-DR, HLA-DQ and HLA-DP. The lack of MHCII expression results in severe immunodeficiency called MHCII deficiency (official WHO nomenclature) or bare lymphocyte syndrome (BLS) (Janeway 2001, Reith and Mach 2001). The effect of PKC activation by TPA on the expression of the IFN-γ-induced HLA-DR class of MHCII was studied in monocytes. IFN-γ (24 hours) induced four-fold induction in HLA-DR expression, whereas TPA treatment for two hours before IFN-γ stimulation resulted in the dramatically decreased expression of HLA-DR. Taken together,

these results indicate that the IFN-γ responsiveness of monocytes is down-regulated by PKC activation through TPA.

8.7. Precise regulation of PKC-δ activation is required for both macrophage differentiation and IFN-γ signaling (I and II)

Jak2 regulates *c-myc* expression which regulates apoptosis in restrictive growth conditions (Askew et al. 1991, Evan et al. 1992, Mohi et al. 1998). PKC-δ also regulates apoptosis as well (Ghayur et al. 1996). The characteristics of apoptosis include cell size contraction, chromatin condensation and DNA digestion (Kerr et al. 1972). The role of Jak2 inhibition by PKC-δ in 32D cells was studied by reversing Jak2 inhibition by ectopically expressing Jak2 in 32D-PKC-δ cells. Initially, these 32D-PKC-δ-Jak2 cells were differentiating normally in response to TPA, but sustained IL-3-mediated Jak2 induction resulted in the apoptosis of the differentiating cells. The apoptosis was more prominent if serum concentration was lower, but it was evident even with normal growth conditions (data not shown).

Tyrphostin AG490, a tyrosine kinase inhibitor which has been suggested to specifically inhibit Jak kinases was used to study whether the inhibition of IL-3-induced Jak2 signaling was enough to induce macrophage differentiation. In thymidine incorporation assays AG490 decreased IL-3-induced DNA synthesis to the level of non-stimulated cells. However, AG490 was unable to induce differentiation, which suggests that growth inhibition was not sufficient for differentiation to proceed but another signal elicited only by PKC-δ in these cells was required for differentiation. As expected, AG490 and TPA both arrested the 32D-PKC-δ cells into the G1 phase of the cell cycle, indicating the importance of the regulation of Jak2 in the progression of the cell cycle (data not shown) (Quelle et al. 1998).

IFN-γ signaling is negatively regulated *in vivo* by SOCS1 and mice lacking SOCS1 expression die perinatally due to enhanced IFN-γ signaling (Krebs and Hilton 2000). In addition, tyrosine phosphatases such as SHP2 play a part in the negative regulation of IFN-γ signaling (Chen et al. 2004). To definitely study the role of PKC-δ on IFN-γ signaling *in vivo* the primary fibroblasts from a wild type mouse and a genetically modified mouse lacking the expression of PKC-δ were used. These mice have an increased B-cell proliferation, an increased amount of autoantibodies, hyperproliferative smooth muscle, and mast cells (Leitges et al. 2001, Tan and Parker 2003). The kinetics of IFN-γ signaling in these cells showed moderately enhanced tyrosine phosphorylation in response to cytokine stimulation. Thus, the effect of PKC-δ depletion on IFN-γ signaling appears to be modulatory, probably reflecting the limited number of circumstances where it is physiologically relevant.

9. DISCUSSION

9.1. PKC as a negative regulator of cytokine signaling

The precise regulation of cytokine signaling is of utmost importance for cellular homeostasis. To secure the adequate regulation of signaling through cytokine receptors, several mechanisms of negative regulation exists. The dysregulation of the signaling pathways of cytokine receptors is associated with various immunological diseases, for example, severe combined immunodeficiency, asthma, and susceptibility to intracellular bacteria infections such as mycobacteria (Shuai and Liu 2003). Primarily, this study set out to investigate more closely the molecular mechanisms of myeloid differentiation and especially the role of PKC in the regulation of macrophage differentiation and activation. IL-3- and IFN-γ-induced signaling was found to be subject to negative regulation through the activation of PKC-δ. In addition, IL-3 and IFN-γ signaling are negatively regulated through the ubiquitination and proteosomal degradation of the key component of these signaling pathways: Jak2.

9.1.1. PKC-δ and IL-3 signaling during the differentiation of macrophages

IL-3 induces the growth and differentiation of the earliest progenitors in the myeloid lineage (Barreda et al. 2004). The macrophage differentiation of 32D-PKC-δ cells is associated with the inhibition of cell growth. Still, the cells require IL-3 in their growth medium. This indicates that IL-3 is not important only for the proliferation but also for the anti-apoptosis and survival of IL-3-dependent cells. The binding of IL-3 to its cognate receptor activates the receptor β-chain-associated Jak2 kinase, followed by Stat5 activation and the induction of the expression of Stat5-regulated genes (Paukku and Silvennoinen 2004). IL-3-induced cellular tyrosine phosphorylation was found to be inhibited by the activation of the ectopically expressed PKC-δ isoform in murine myeloid progenitor cells, 32D. Especially affected appeared to be phosphoproteins around 120-140 kDa. Jak2 was identified as a target for PKC-δ mediated serine/threonine phosphorylation resulting in the decreased tyrosine phosphorylation and activation of Jak2.

Different signaling pathways regulate various cellular responses such as apoptosis and proliferation (Kinoshita et al. 1995). The inhibition of IL-3-

induced *c-myc* results in decreased proliferation but does not remove the protection against apoptosis (Kinoshita et al. 1995). The expression of *c-myc* is regulated by Shc adaptor protein (Gotoh et al. 1996). The inhibition of IL-3/Stat5 activated genes through the expression of C-terminally truncated Stat5 demonstrated that the cis, osm and pim-1 genes were profoundly inhibited by dysfunctional Stat5, and that *c-fos* was partially inhibited as was cellular growth (Mui et al. 1996). In line with these studies, Shc phosphorylation was not altered in response to TPA either in 32D parental or 32D-PKC-δ cells. This suggests that Jak2 activity does not regulate Shc activity in IL-3 signaling, and that PKC-δ-mediated effect on cell growth occurs probably through the inhibition of Stat5 leading to partial decrease in *c-fos* expression and not through *c-myc* inhibition.

Src protein kinases have been suggested to regulate IL-3-induced Stat3 phosphorylation and growth signaling whereas Jak2 would regulate antiapoptotic signals (Chaturvedi et al. 1998). However, non-cytokine activated Jak2 can up-regulate the *c-myc* gene during Bcr-Abl oncoprotein expression through non-Stat5-mediated fashion (Xie et al. 2002). Interestingly, during the preparation of this work, a single mutation was characterized in the pseudokinase domain of Jak2 resulting in a constitutively active form of Jak2 and the induction of polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis (Baxter et al. 2005, James et al. 2005, Kralovics et al. 2005). This acquired mutation also led to the IL-3-independent growth of cultured Ba/F3 and FDCP1 cells (Kralovics et al. 2005). It is still unclear how the V617F mutation of the JH2 domain in Jak2 causes increased cell proliferation and survival in the absence of IL-3, but increased Jak2 and Stat5 tyrosine phosphorylation were observed (Kralovics et al. 2005). Taken together the results discussed above, it could be envisioned that Jak2 might Stat5-independently activate *c-myc* and Stat5-dependently participate in *c-fos* induction. These findings also emphasize the importance of the precise regulation of Jak2 functions in hematopoietic cells. It is still somewhat unclear why Jak2 activation induced by the GM-CSF receptor, which shares common signaling β-chain with IL-3 and IL-5 is not affected by PKC activation. This discrepancy might be explained by inadequate receptor α-chain expression in the cell lines studied (HL-60, THP-1, U-937) (Byrne 1989), even though we did detect moderate Jak2 tyrosine phosphorylation in response to GM-CSF in these cells.

Jak inhibitor AG490 inhibited IL-3-induced growth and DNA synthesis but did not induce the macrophage differentiation of 32D-PKC-δ cells. PKC-δ expression and activation in 32D cells similarly resulted in growth inhibition but it also induced differentiation. Thus, PKC-δ appears to induce two different signals in differentiating macrophages. It promotes the inhibition of IL-3 growth signaling through Jak2 but it also induces the expression-specific set of genes required for macrophage phenotype via an unknown mechanism.

Classical, novel and atypical PKCs regulate the activity of the MAPK/ERK pathway and it could be envisaged that the PKC-δ-mediated inhibition of Jak2 might be regulated through MAPK/ERK activation (Schonwasser et al. 1998). Several findings argue against the significant role of MAPK/ERKs in PKC-

δ/Jak2 regulation. First of all, by using a specific inhibitor of mitogen-activated ERK-activating kinases 1/2 (MEK-1/2), the kinases which are the upstream activators of ERK1 and ERK2 (Schonwasser et al. 1998), the TPA-mediated inhibition of Jak2 in 32D-PKC-δ cells was not reversed. However, the inhibitor used (PD098059) prevents MEK activation by Raf. It associates to inactive MEK and inhibits Raf from phosphorylating it but, importantly, PD098059 has no effect on active MEK thus making it possible that a fraction of activated MEK might regulate Jak2 activity, but PKC-δ would not play a role in the process. Still, the lack of isoform specificity in MEK1/2-mediated PKC activation (Schonwasser et al. 1998) also argues against the role of the MAPK/ERK pathway in PKC-isoform-specific inhibition of Jak2, described in this work. Finally, co expression studies and PKC *in vitro* kinase assays indicate a clear interaction of the kinases suggesting that Jak2 might be a PKC-δ target also *in vivo*.

9.1.2. PKC-δ as a negative regulator of IFN-γ-induced macrophage activation

Interferon-γ is produced mainly by CD4 positive Th_1-cells, CD8 positive T-cells and NK cells. The main function of this cytokine is macrophage activation (Janeway 2001). The role of IFN-γ *in vivo* has been studied in genetically modified mice lacking a component of the IFN-γ signal transduction pathway. These studies have revealed that mice lacking functional IFN-γ, IFN-γR or Stat1 are more susceptible to infections by parasites, bacteria and viruses (Dorman and Holland 2000). On the other hand, the ectopic expression of IFN-γ in transgenic mice results in a greatly reduced number of B-cells, a two-fold decrease in myeloid progenitors and 50% decrease in T-cells in the spleen, indicating various immunological disturbances (Young et al. 1997). It is important to keep in mind that despite the fact that genetically modified mouse models are good tools in studying the regulation of cytokine signaling, severe restrictions also exist in extrapolating these results from mice to humans. The genetical background of mice in these studies have been different, and recently a study suggested that in mice with the same genetical background the lack of IFN-γ receptor chain was more lethal than the lack of IFN-γ itself (Cantin et al. 1999). A genetically modified phenotype may result from the developmental effects of null mutation or the lack of a functional gene in adult tissue (Tan and Parker 2003). However, recently described mutations in the IFN-γ signaling of humans have been described, and it appears that the carriers of these mutations are prone to intracellular bacterial infections, such as mycobacterial infections (Dupuis et al. 2001). The aforementioned results clearly show that IFN-γ signaling requires tight regulation.

Based on the data presented in this study, PKC-δ has a modulatory role in the negative regulation of IFN-γ signaling as shown by the experiments performed in fibroblasts from mice lacking PKC-δ expression. Mice lacking PKC-δ are viable,

but they have increased B-cell numbers and a dysregulated apoptosis of SMC, indicating a role for PKC-δ in both the regulation of proliferation and apoptosis (Leitges et al. 2001, Miyamoto et al. 2002). In contrast, the lack of expression of the far more potent regulator of IFN-γ signaling, SOCS1, results in perinatal lethality and a phenotype similar to IFN-γ administration (Gresser et al. 1975, Starr et al. 1998).

To underscore the importance of the tight regulation of IFN-γ signaling, positive regulators of IFN-γ signaling have also been identified. The lack of integrin-mediated PKC-ε activation leads to decreased IFN-γ responses (Ivaska et al. 2003). Even more interestingly, PKC-ε is also a critical molecule for macrophage activation and defence against bacterial infections, and the lack of this kinase leads to impaired IFN-γ responses (Castrillo et al. 2001). The identification of Jak2 as a target for negative regulation through the activation of PKC-δ adds one more layer to the complexity of the negative regulation of cytokine signaling. As in the case of IL-3 discussed above, different mechanisms for the PKC-δ-mediated inhibition of Jak2 could be envisaged. Especially important was to assess the role of phosphatases, such as SHP2 in IFN-γ-induced signaling, since they play a role in both the constitutive and the cytokine-inducible regulation of IFN-γ signaling (Haque et al. 1995, You et al. 1999). Also, TPA induces transmembrane tyrosine phosphatase PTPα activity through PKC-δ activation (Brandt et al. 2003). Phosphatases appear to have no major role in PKC-δ-mediated Jak2 inhibition, since a phosphotyrosine phosphatase inhibitor such as Na_3VO_4 was unable to reverse Jak2 inhibition in response to PKC-δ activation.

TPA also depletes PKC in cells, and therefore it was important to use short (30 minutes) TPA treatments to avoid PKC depletion. 30 minutes of TPA treatment had no effect on PKC-δ-levels in any of the cell lines used in this study, indicating that the results observed were not *de facto* caused by decreased PKC-δ activity.

In addition to the pharmacological activation of PKC-δ through TPA, PKC-δ activation was induced through the ligand binding and activation of FcγR. Fc receptors activate macrophages (Gordon 2003), and the FcγR-mediated activation of macrophages appears to direct immune responses towards Th_2 (Anderson et al. 2004). The observed PKC-δ activation was rapid and transient, resulting in the inhibition of IFN-γ signaling in 20 minutes, even though a decrease in signaling was detectable already after 10 minutes. This would strongly argue against involvement of the SOCS proteins in this rapid inhibition of IFN-γ signaling, because SOCS1 expression by for example IFN-γ or immunocomplexes requires at least 30 minutes (Sakamoto et al. 1998, Gomez-Guerrero et al. 2004).

IL-3 and IFN-γ share an immediate signaling molecule, Jak2. However, the effects of these cytokines especially on cell growth are opposed to each other. IL-3 induces the proliferation of myeloid precursor cells whereas IFN-γ has an antiproliferative effect on macrophages (Boehm et al. 1997, Barreda et al. 2004). It is important to distinguish between the Stats activated by Jak2 to fully

understand the seemingly controversial results. Stat1 is considered as a tumor suppressor due to its inhibitory effects on growth, whereas Stat5 possesses transforming properties and is strongly associated with tumor development and progression (Durbin et al. 1996, Bowman et al. 2000). Thus, the inhibition of Jak2 by PKC-δ appears to serve as an important regulatory mechanism utilized upon specific cellular requirements. In addition, the reversal of Jak2 inhibition during macrophage differentiation results in the apoptosis of differentiating cells. This further underscores the importance of the precise and balanced regulation of cytokine signaling.

9.2. PKC as a positive regulator of cytokine signaling

As discussed above, IL-3-induced growth signaling is inhibited by PKC-δ. The inhibition of growth is not enough to induce macrophage differentiation. Thus, an additional signal has to be delivered by PKC-δ to up-regulate macrophage-phenotype-associated genes.

9.2.1. M-CSF-induced differentiation and PKC-δ

Cytokines regulating terminal macrophage differentiation include GM-CSF and the polypeptide growth factor M-CSF. GM-CSF also induces granulocyte differentiation, whereas M-CSF solely induces the phenotype of mature macrophages (Barreda et al. 2004). Early studies on the M-CSF receptor indicated that PKC is activated upon M-CSF stimulation (Imamura et al. 1990). At the time these preliminary findings were made, only few PKC isoforms had been identified. Since then, isoform specificity upon M-CSF-induced PKC activation has not been elucidated in detail even though the number of identified PKC isoforms has multiplied. However, it has been shown that M-CSF induces PKC-α expression in addition to its nuclear translocation in granulocyte macrophage colony-forming cells (GM-CFC) which may develop into either macrophages and/or neutrophils. Additionally, PKC-α regulates macrophage differentiation in 32D-PKC-α cells and GM-CFC cells (Mischak et al. 1993, Whetton et al. 1994, Pierce et al. 1998). PLC-γ2 is rapidly activated by M-CSF (Bourette et al. 1997). PLC-γ2 is a possible upstream activator of the classical and novel PKC isoforms because it participates in the hydrolysis of phosphatidylinositol, resulting in DAG formation. DAG in turn binds to the DAG binding domain in the regulatory domain of cPKCs and nPKCs (Newton 1997). PKC-δ, a novel PKC isoform was rapidly activated by M-CSF as detected by several methods.

The mutation of the autophosphorylation site tyrosine 807 of M-CSFR (also known as Fms) abrogates M-CSF-induced differentiation (Bourette et al. 1995). PKC-δ activation was decreased in cells expressing M-CSFR-Y807 mutation.

Furthermore, the mutation of tyrosine 721, which supports but does not regulate differentiation (Bourette et al. 1995), decreases PKC-δ activation to a lesser extent than tyrosine 807 mutation, which suggests that PKC-δ activity plays a key role in the induction of macrophage differentiation in murine cells. This is also in line with the the fact that tyrosine 807 binds and activates PLC-γ (Bourette et al. 1997). This would suggest a step-by-step activation cascade in response to M-CSFR activation, where PLC-γ binds to M-CSFR and becomes activated, and subsequently induces PKC-δ activity.

PKC-δ was hypothesized to regulate downstream genes involved in the terminal differentiation of macrophages. A possible target gene, PRKX, is induced by PKC-β in human HL-60 cells and it is expressed mainly in granulocytes and macrophages (Semizarov et al. 1998). The PRKX structure resembles the catalytic subunit of cyclic adenosine monophosphate (cAMP) dependent protein kinases, such as protein kinase A (PKA) (Klink et al. 1995). cAMP-dependent protein kinases are activated upon cAMP binding to their regulatory subunits, which releases a catalytic subunit to function as a serine/threonine kinase on substrate molecules (Chin et al. 2002). Correspondingly, PRKX activity is regulated by cAMP (Zimmermann et al. 1999). Transcriptional effects mediated by cAMP-regulated kinases occur via the phosphorylation of the common transcription factor, cAMP response element binding protein (CREB). Interestingly, PRKX is involved in CREB-mediated transcription (Di Pasquale and Stacey 1998). Since the human homologue of Pkare, PRKX, is up-regulated specifically during myeloid differentiation and Pkare contains several conserved amino acids required for the PKA function, it could be envisaged that macrophage differentiation up-regulates the expression of Pkare. The induction of Pkare expression in FDCP1-Fms-WT cells in response to M-CSF occurs slowly, as compared to the 32D-PKC-δ response to TPA. This probably reflects the slower induction of the macrophage phenotype in FDCP1-Fms-WT cells. In 32D-PKC-δ cells, TPA stimulation of 6 hours renders the cells adherent, whereas 24-36 hours of M-CSF stimulation is required for increased adherence in FDCP1-Fms-WT cells.

It could be envisaged that Pkare might act as a differentiation-induced regulator of transcription by regulating a set of genes through CREB activation. Nevertheless, Pkare expression is associated with macrophage phenotype, suggesting specific functions for this gene in myeloid differentiation. However, it is important to remember that in addition to our results on Pkare, only one functional report (Blaschke et al. 2000) has been published about this gene to date (September 2005), leaving the definitive functions of this kinase to be elucidated. In addition, the direct extrapolarization of results obtained in mice to humans has its limitations as has been discussed above in context of PKC-deficient mice. In the case of PRKX, this kinase which is located in the X-chromosome (Xp22.3) has a counterpart in the Y-chromosome: PRKY (Yp11.2) (Schiebel et al. 1997). Importantly, Pkare is located close to the centromer of the mouse X-chromosome but it does not have a homologue in the Y-chromosome, rendering the male mice hemizygous for this gene. Thus, between humans and

mice, several differences between PRKX and Pkare exist, including the chromosome localization and the lack of an Y-chromosomal homologue of Pkare (Blaschke et al. 2000). Future experiments involving the genetical modification of mice to yield constitutional and conditional Pkare $^{-/-}$ mice as well as transgenic mice expressing Pkare under hematopoietic transcription factors are required to determine the downstream targets of this novel kinase.

9.3. Inhibition of cytokine signaling by the ubiquitin-proteasome pathway

Jak2-mediated signaling in hematopoietic cells requires precise regulation as shown by conditions with the constitutively active expression of Jak2 resulting in cytokine-independent proliferation and malignancies (Lacronique et al. 1997, Kralovics et al. 2005). One mechanism for negative Jak2 regulation might be its degradation. Protein degradation through proteasomes ensures the removal of unnecessary proteins but it also serves as an irreversible regulator of signaling by rapidly destroying critical signaling molecules (Glickman and Ciechanover 2002).

9.3.1. Regulation of cytokine-induced Jak2 via ubiquitination

Ubiquitin-proteasome-mediated degradation of proteins is based on the ubiquitination (conjugation of multiple ubiquitin moieties) of target proteins which leads to the recognition of polyubiquitinated proteins by proteasome 26S complex which ultimately leads to the degradation of the tagged protein (Ciechanover et al. 2000). The irreversibility of degradation requires the tight regulation of its initiation. Ubiquitin ligation is regulated by three enzymes (E1, E2, and E3). Only one E1 enzyme has been discovered, whereas numerous E2 and E3 enzymes have been described (Glickman and Ciechanover 2002). The existence of several E2 and E3 ligases allow substrate specificity in the regulation of protein degradation. Post-translational modification, phosphorylation, plays an important part in the ubiquitin-mediated degradation of several important regulator proteins, including Src-kinases, IκB-α and *c-Jun* (Laney and Hochstrasser 1999). IL-3 induced Jak2 polyubiquitination but polyubiquitination did not have an effect on Jak2 protein levels as detected by Western blotting. Tyrosine phosphorylation was important for Jak2 polyubiquitination. On the other hand, the co expression of Jak2 and PKC-δ decreased Jak2 tyrosine phosphorylation but had no effect on Jak2 ubiquitination. This might suggest that the PKC-δ-mediated inhibition of Jak2 tyrosine phosphorylation might actually protect Jak2 from polyubiquitination and thus degradation. These findings also imply that the tyrosine phosphorylated and ubiquitinized Jak2 fraction in cells upon IL-3 stimulation is small compared

to the total amount of Jak2 in cells. Thus the sensitivity of Western blotting is not enough to detect the decrease in Jak2 protein levels. In the case of IFN-γ-induced Jak2 polyubiquitination, the same effect was detected, suggesting that cytokine-induced tyrosine phosphorylation and ubiquitination only involves a small but functionally important fraction of total cellular Jak2 protein. These results were confirmed by experiments performed with SOCS1 $^{-/-}$ thymocytes, where Jak2 protein levels were not markedly affected by IFN-γ treatment, but IFN-γ-induced complexes with high-molecular weight reactive to anti-ubiquitin were clearly decreased in SOCS1 $^{-/-}$ cells (data not shown).

SOCS1 is a regulator of IFN-γ signaling and phosphorylated Jak2 (Giordanetto and Kroemer 2003, Ilangumaran et al. 2004). The interaction of Jak2 and SOCS1 was prolonged upon treatment with protease inhibitors, suggesting an important role for SOCS1 in the ubiquitin-mediated degaradation of Jak2. The exact mechanism of SOCS1 involvement in Jak2 ubiquitination and degaradation is still uncelar. SOCS proteins contain a conserved domain, the SOCS-box, which binds to elongin B/C (Zhang et al. 1999) which may target proteins to degradation through a protein complex similar to the von Hippel-Lindau tumor suppressor E3 ligase complex. This complex also contains a bridging factor, RING finger protein Rbx1 and Cullin-2. It will be of interest to learn whether the SOCS-protein-associated degradation complex involves the same proteins (Duan et al. 1995, Kibel et al. 1995).

Considering the inhibitory mechanisms of Jak2 discussed above, it could be envisaged that Jak2 regulation by PKC-δ and ubiquitination occurs subsequently. The rapid and reversible PKC-δ-mediated inhibition of tyrosine phosphorylation is obtained within minutes, whereas the ubiquitination and irreversible degradation of protein would ensure the down-regulation of Jak2-mediated signaling should tyrosine phosphorylated molecules appear after PKC-δ activation. However, it should not be forgotten that parallel regulatory mechanisms exists, and future studies will probably reveal more interactions between these different mechanisms.

10. SUMMARY AND CONCLUSIONS

Cytokines play a central role in hematopoiesis and immune responses. During the past decades our understanding on the mechanisms of the intracellular actions of cytokines has increased markedly. The characterization of a central mechanism in cytokine signaling, the Jak/Stat pathway, has revealed that four Jaks and seven Stats specifically regulate the expression of genes central in many physiological processes. Currently, growing interest has focused on factors negatively regulating the Jak/Stat pathway, as the capability to inhibit these signaling molecules could potentially provide new therapeutic strategies for a number of human ailments such as asthma, immunodeficiencies and hematopoietic malignancies (Bromberg 2002, Candotti et al. 2002, Pernis and Rothman. 2002).

Eventually, this study sought to characterize the mechanism of growth inhibition during macrophage differentiation. A novel inhibitory mechanism of Jak2 through serine/threonine phosphorylation was discovered. Serine phosphorylation may have totally different effects depending on the target protein. In the case of Stat1, serine phosphorylation further activates the protein and it is required for the full transcriptional activity of Stat1 by recruiting positive transcriptional cofactors (Zhang et al. 1998). The PKC-δ-mediated inhibition of the Jak2 kinase was observed after both IL-3- and IFN-γ-induced Jak2 activation. As expected, the inhibition of Jak2 led to decreased downstream Stat activation. Imbalanced Jak2/PKC-δ activation led to apoptosis in differentiating macrophages. Additionally, a novel mechanism for Jak2 inhibition was discovered to result from the increased ubiquitination and degradation of tyrosine phosphorylated Jak2. In addition to pharmacological activation, PKC-δ was activated by M-CSF and FcγR. In the FDCP1 murine macrophage differentiation model, M-CSF-induced PKC-δ activity correlated to the differentiation responses, in other words, M-CSF failed to activate PKC-δ in cells carrying the mutated M-CSF receptor unable to induce differentiation.

Based on these results, interesting questions for future studies can be envisaged. First of all, the mechanism of the apoptosis of differentiating macrophages subjected to the overexpression of Jak2 and thus enhanced IL-3 stimulation is still unclear. Since neither TPA-induced PKC-δ nor IL-3-activated Jak2 alone induce apoptosis in 32D or 32D-PKC-δ cells, the simultaneous activation of both has to dysregulate the downstream signaling via an unknown mechanism which leads to apoptosis. Interestingly, in response to H_2O_2, Jak2

appears to be proapoptotic in the smooth muscle cells of rats (Sandberg and Sayeski 2004) whereas the lack of PKC-δ reduces apoptosis in the smooth muscle cells of mice, suggesting an apoptotic role also for PKC-δ (Leitges et al. 2001).

Taking into account the recently-described point mutation of the Jak2 pseudokinase domain which leads to constitutively active Jak2 (James et al. 2005), it would be worthwhile to further characterize the molecular interaction of Jak2 and PKC-δ also on the amino acid level. The utilization of bioinformatics by algorithms predicting possible PKC-δ phosphorylation sites (Fujii et al. 2004) combined with classical PCR-based mutagenesis will provide powerful tools for these studies.

M-CSF-induced differentiation signaling is mediated by PKC-δ isoform. Comparative studies of gene expression in cells with the M-CSF wild-type receptor or a receptor mutant unable to induce differentiation (Y807) (Bourette et al. 1995) using DNA-microarray technology should be informative in characterising the exact genes required for macrophage differentiation. This information could be then used for gene-specific studies on individual, differentiation-associated genes and in the gene therapy of diseases with dysregulated myleoid differentiation, such as acute myeloid leukemia.

This study has revealed novel mechanisms of negative regulation in IL-3 and IFN-γ signaling by characterising the serine/threonine phosphorylation and the polyubiquitination of Jak2 as important regulatory mechanisms of activated Jak2. A detailed understanding of these regulatory pathways will yield improved abilities to therapeutically interfere with diseases associated with the dysregulated signaling of not only IL-3 and IFN-γ but also by other cytokines that utilizing Jak/Stat signaling.

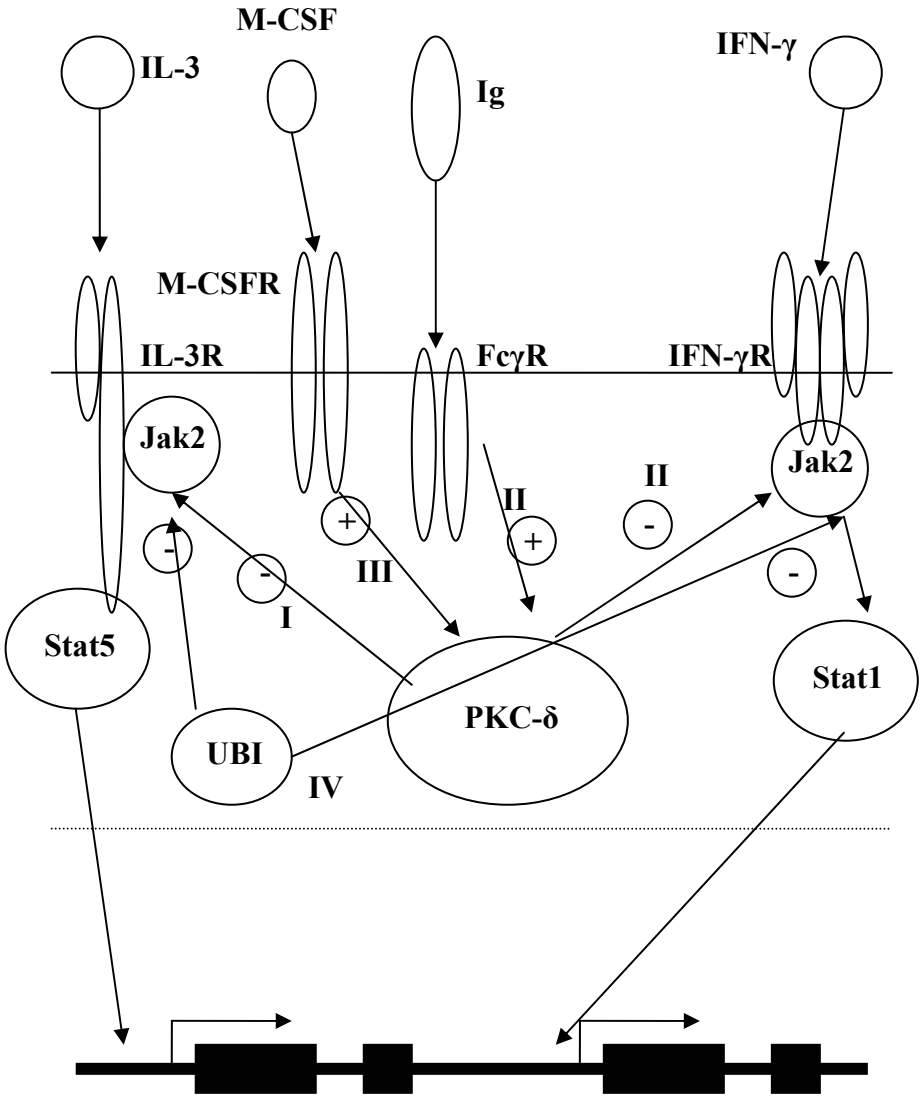

Figure 5. Main findings of this study. Roman numerals (I-IV) refer to original communications.

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