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Expression and activation of STAT and IRF family transcription factors in mononuclear leukocytes

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2005

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EXPRESSION AND ACTIVATION OF STAT AND IRF FAMILY TRANSCRIPTION FACTORS IN MONONUCLEAR LEUKOCYTES

A C A D E M I C D I S S E R T A T I O N

To be presented with the permission of the Faculty of Biosciences, University of Helsinki, for public examination in the Arppeanum auditorium, Helsinki University Museum, Snellmaninkatu 3, Helsinki, on January 14th, at 12 o'clock noon.

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ABSTRACT

The immune system protects the host from microbial attacks and malignant transformation. The antimicrobial defence system consists of innate and adaptive immune responses. The innate system is activated by recognition of conserved structures found in pathogens but not in the host itself. Monocyte/macrophages, natural killer (NK) cells, and dendritic cells (DCs) are key players in the innate immune response. Macrophages directly phagocytose and destroy infectious agents, and NK cells destroy virus-infected or malignant cells by cytolysis. DCs capture and process microbe-derived foreign antigens. Subsequently they move to local lymph nodes to present the antigens to naive T cells, thus activating antigen-specific T and B cell responses. During the innate response macrophages, NK cells, and DCs produce cytokines that guide the ongoing innate immune responses, and stimulate the initiation of the adaptive immune response.

Cytokines are a group of intercellular signaling proteins that regulate cellular proliferation, differentiation, and immune responses. The extracellular cytokine signal from the cell surface receptor into the nucleus is transmitted via receptor-coupled signal transduction systems. The Janus kinase–signal transducer and activator of transcription (JAK–STAT) pathway is the system most widely used by cytokines. Other signaling pathways and transcription factors are also activated by cytokines, including the interferon regulatory factors (IRFs). The signals from all activated pathways converge in the nucleus. In eukaryotic cells, the availability of regulatory DNA elements to transcription factors and the transcriptional machinery is regulated by covalent modification of DNA-associated histone proteins and, in some cases, the DNA itself.

Receptors, signalling proteins, and transcription factors are the molecules that enable the cells to interpret incoming signals and respond to these signals by regulated changes in their transcriptional profiles. These changes can for example lead to secretion of cytokines or other signaling proteins or enhance proliferation of the cell. During cellular differentiation, various genes are expressed in a stage-specific

manner, and abrupt changes in the expression levels of given genes can occur when the cells shift from one developmental stage to another.

In this work, expression and activation of STAT and IRF family transcription factors was studied in human immune cells. This work shows that interferon (IFN) stimulation increased the expression of signalling components essential for IFN responses, Stat1, Stat2, and IRF9, leading to an enhanced IFN-response. IFNs are cytokines produced by cells during microbial infections. This kind of a positive feedback mechanism may be one means by which the IFN-system strengthens the innate immune response. Additionally, differentiation of monocytes into macrophages was characterized. Differentiation was found to significantly alter the cells' responsiveness to the growth-promoting cytokine granulocyte/macrophage colony-stimulating factor (GM-CSF). Significantly reduced activation of Stat5 by GM-CSF and decreased expression of its target genes in macrophages was detected. Decreased Stat5 activation in macrophages may be one mechanism by which the expression of monocyte-specific genes is prevented when the cells differentiate. In this work it is also shown that in NK and T cells, cytokines induced the expression of IRF1, IRF4, and IRF8 transcription factors. IRF4 expression was regulated by Stat4, and a Stat4-binding DNA-element was described in the IRF4 promoter region. STAT and IRF expression was also studied in monocytes, macrophages, and DCs. IRF4 and Stat4 transcription factors were found to be constitutively expressed in DCs but not in macrophages. The functionality of several IRF4 promoter DNA-elements was also characterized in DCs.

This thesis work provides information on how cytokines regulate the functions of immune cells. Regulation of transcription factor expression and activation in cells ensures that cell type-specific cytokine responses are initiated in an orderly fashion. Ultimately, the interplay between STATs and IRFs allows the cells to prolong and fine-tune the effect of the cytokine signals they receive.

Keywords: immune system, leukocyte, cytokine, gene regulation, transcription factor, STAT, IRF

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TIIVISTELMÄ

Immuunijärjestelmän tehtävänä on suojella yksilöä taudinaiheuttajilta ja pahanlaatuisilta solumuutoksilta. Immuunivaste jakautuu synnynnäiseen ja hankittuun immuniteettiin. Monosyytit, makrofagit, dendriitti- ja NK-solut ovat veren valkosoluja, joilla on synnynnäisessä immuunivasteessa tärkeitä tehtäviä. Makrofagit fagosytoivat ja tuhoavat taudinaiheuttajia, ja NK-solut tuhoavat virusten infektoimia tai pahanlaatuisiksi muuntuneita soluja. Dendriittisolut esittelevät vieraat mikrobiantigeenit T- ja B-soluille aikaansaaden antigeenispesifisen hankitun immuniteetin aktivoitumisen. Aktivoituneet monosyytit, makrofagit, dendriitti- ja NK-solut tuottavat liukoisia välittäjämolekyylejä, sytokiineja, jotka säätelevät solujen kasvua ja erilaistumista sekä tulehdusreaktioiden ja immuunipuolustuksen aktivoitumista. Sytokiinien signaalinvälitys tapahtuu tarkoin säädellysti. Sytokiinisignaali siirtyy solun sisällä useimmiten JAK–STAT-reitin välityksellä. STAT-proteiinit ovat sytoplasmisia transkriptiotekijöitä, jotka aktivoituvat sytokiinin sitoutuessa reseptoriinsa, muodostavat keskenään dimeerejä ja siirtyvät tumaan. Tumassa ne säätelevät sytokiinien kohdegeenien ilmentymistä. Sytokiinit aktivoivat myös muita signaalireittejä ja säätelyproteiineja, kuten IRF-transkriptiotekijöitä. Eukaryoottisoluissa geenien ilmentymistä säädellään myös muokkaamalla DNA:ta sitovia histoniproteiineja. Näin säädellään sitä, kuinka helposti transkriptiotekijät pääsevät sitoutumaan kohteenaan oleviin DNA-elementteihin geenien säätelyalueilla.

Solujen toiminnan kannalta on tärkeää, mitä pintareseptoreja, signaalinvälitys-molekyylejä ja transkriptiotekijöitä solussa ilmennetään, sillä näiden molekyylien avulla solu kykenee muuntamaan ympäristöstä tulevat signaalit muutoksiksi solun geenien ilmentymisessä. Muutos geenien ilmentymisprofiilissa vaikuttaa solun toimintaan, ja voi esimerkiksi saada aikaan tiettyjen proteiinien erittämisen solusta tai muutoksia solun kasvupotentiaalissa. Esimerkiksi erilaistumisen aikana soluissa ilmennetään kussakin kehitysvaiheessa tarvittavia proteiineja, ja eriasteisesti erilaistuneiden solujen geenien ilmentymisessä voi olla suuriakin eroja.

Tässä väitöskirjatyössä on tutkittu STAT- ja IRF-perheen transkriptiotekijöiden ilmentymistä ja aktivaatiota ihmisen immuunijärjestelmän soluissa. Ensimmäisessä osatyössä havaittiin, että interferonistimulaatio voimisti interferonien signaalinvälitykseen osallistuvien transkriptiotekijöiden, Stat1:n, Stat2:n ja IRF9:n, ilmentymistä makrofageissa, mikä johti voimistuneeseen interferonivasteeseen. Interferonit ovat sytokiineja, joita solut tuottavat infektioiden yhteydessä. Tällaisen positiivisen takaisinkytkentämekanismin avulla solut voivat tehokkaammin taistella taudinaiheuttajia vastaan, ja se voi olla yksi tapa, jolla interferonit voimistavat synnynnäistä immuniteettia. Toisessa osatyössä tarkasteltiin monosyyttien erilaistumista makrofageiksi. Tässä työssä havaittiin, että monosyyttien ja erilaistuneiden makrofagien vaste GM-CSF-kasvutekijälle on erilainen. GM-CSF aktivoi Stat5-transkriptiotekijän monosyyteissä voimakkaammin kuin makrofageissa. Samoin Stat5:n aktivoimien kohdegeenien ilmentyminen monosyyteissä oli tehokkaampaa kuin makrofageissa. Stat5-aktivaation vaimentuminen liittyi erilaistumiseen monosyytistä makrofagiksi. Tämä saattaa olla eräs mekanismi, jolla estetään monosyyttispesifisten geenien ilmentyminen makrofageissa. Kolmannessa osatyössä tarkasteltiin IRF-transkriptiotekijöiden ilmentymistä ihmisen NK- ja T-soluissa. IRF4 on T-solujen erilaistumiselle ja toiminnalle välttämätön proteiini. Sen ilmentyminen aktivoituu, kun T-solu tunnistaa spesifisen antigeeninsa. Tämän työn päähavainto oli, että myös sytokiinit voimistavat IRF4-transkriptiotekijän ilmentymistä NK- ja T-soluissa. Tämä tapahtuu Stat4-transkriptiotekijän välityksellä, ja työssä kuvattiin myös IRF4-geenin säätelyalueelta DNA-elementti, johon Stat4-proteiini sitoutuu. Neljännessä osatyössä tutkittiin STAT- ja IRF-transkriptiotekijöiden ilmentymistä ja aktivaatiota monosyyteissä, makrofageissa ja dendriittisoluissa. Työssä havaittiin, että Stat4 ja IRF4 ilmentyvät dendriittisoluissa, mutta eivät makrofageissa. Lisäksi työssä karakterisoitiin IRF4-geenin säätelyalueelta useita eri transkriptiotekijöitä sitovia DNA-elementtejä ja analysoitiin niiden toimivuutta IRF4-geenin ilmentymisen säätelyssä dendriittisoluissa.

Tämä väitöskirjatyö syventää tietämystä siitä, kuinka sytokiinit säätelevät immuunipuolustuksen kannalta tärkeiden solujen toimintaa. Säätelemällä transkriptiotekijöiden ilmentymistä ja aktivaatiota soluissa varmistetaan, että oikea-aikainen, solytyyppikohtainen sytokiinivaste käynnistyy tarkoituksenmukaisella tavalla immuunivasteen aikana.

Avainsanat: immuunijärjestelmä, verisolut, välittäjäaine, geeninsäätely, transkriptiotekijä

CONTENTS

ABBREVIATIONS

APC	antigen presenting cell
CBP	CREB binding protein
CD	cluster of differentiation
cDNA	complementary DNA
ChIP	chromatin immunoprecipitation
CML	chronic myeloid leukemia
CNTF	ciliary neurotrophic factor
CREB	cyclic AMP response element binding protein
CSF	colony stimulating factor
CTL	cytotoxic T lymphocyte
DC	dendritic cell
EGF	epidermal growth factor
EMSA	electrophoretic mobility shift assay
EPO	erythropoietin
ERK	extracellular signal regulated kinase
FCS	fetal calf serum
FGF	fibroblast growth factor
GAS	IFN-γ activated sequence element
G-CSF	granulocyte colony stimulating factor
GH	growth hormone
GM-CSF	granulocyte-macrophage CSF
HAT	histone acetyl transferase
HDAC	histone deacetylase
HMG	high mobility group
HTLV	human T cell leukemia virus

Ig	immunoglobulin
IFN	interferon
IKK	inhibitory κB kinase
IL	interleukin
IRF	interferon regulatory factor
ISGF	interferon stimulated gene factor
ISRE	interferon stimulated response element
IU	international unit
JAK	Janus tyrosine kinase
JNK	c-Jun N-terminal kinase
LIF	leukemia inhibitory factor
LPS	lipopolysaccharide
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinase
M-CSF	macrophage colony stimulating factor
MHC	major histocompatibility complex
mRNA	messenger RNA
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor-κB
NK	natural killer
OSM	oncostatin M
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCAF	p300/CBP associated factor
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor

PIAS	protein inhibitor of activated STATs
PRL	prolactin
RT-PCR	reverse transcriptase-PCR
SDS	sodium dodecyl sulphate
SH2	src homology region 2
SHP-1	SH2 domain-containing phosphatase-1
SOCS	suppressor of cytokine signaling
STAT	signal transducer and activator of transcription
SUMO	small ubiquitin-like protein modifier
TCR	T cell receptor
TGF	transforming growth factor
Th	T helper
TLR	Toll-like receptor
TNF	tumor necrosis factor
TPO	thrombopoietin

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by their Roman numerals. Some unpublished data is also presented.

I Lehtonen A, Matikainen S, Julkunen I. Interferons up-regulate STAT1, STAT2, and IRF family transcription factor gene expression in human peripheral blood mononuclear cells and macrophages. Journal of Immunology, 159:794-803, 1997.

II Lehtonen A, Matikainen S, Miettinen M, Julkunen I. Granulocyte-macrophage colony stimulating factor (GM-CSF)-induced STAT5 activation and target-gene expression during human monocyte/macrophage differentiation. Journal of Leukocyte Biology, 71:511-519, 2002.

III Lehtonen A, Lund R, Lahesmaa R, Julkunen I, Sareneva T, Matikainen S. IFN-α and IL-12 activate IFN regulatory factor 1 (IRF-1), IRF-4, and IRF-8 gene expression in human NK and T cells. Cytokine, 24:81-90, 2003.

IV Lehtonen A, Veckman V, Nikula T, Lahesmaa R, Kinnunen L, Matikainen S, Julkunen I. Regulated expression of interferon regulatory factor (IRF) 4 during differentiation of human dendritic cells and macrophages. Submitted for publication.

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1 INTRODUCTION

The immune response can be divided into two arms: innate and adaptive responses. The innate system provides the first line of defence against pathogens. The activation of innate responses relies on recognition of conserved structures found in pathogens, called pathogen-associated molecular patterns, to distinguish between self and the invading microorganism. This recognition is mainly mediated by Toll-like receptors (TLRs) present on the cell surface of dendritic cells (DCs) and macrophages. Monocyte/macrophages and natural killer (NK) cells function in the heart of the innate immune response. Macrophages act as scavengers by directly phagocytosing and destroying infectious agents, and NK cells destroy virus-infected or malignant cells by cytolysis. DCs are antigen-presenting cells that capture and process microbe-derived foreign antigens. Subsequently they move to local lymph nodes to present antigens to naive T cells, thus activating antigen-specific immune responses. Macrophages, NK cells, and DCs produce cytokines that guide the ongoing innate immune responses, and stimulate the initiation of the adaptive immune response by T and B cells, the cell types able to recognize and respond to specific antigens.

Cytokines are a group of intercellular signaling proteins that play important roles in regulating cell homeostasis and immune responses. The extracellular cytokine signal has to be transmitted from the cell surface into the nucleus via receptor-coupled signal transduction systems. The Janus kinase–signal transducer and activator of transcription (JAK–STAT) pathway is the system most widely used by cytokines. Other signaling pathways and transcription factors activated by cytokines include the mitogen-activated protein kinase (MAPK) pathway, the nuclear factor (NF)-κB-system, and the interferon regulatory factors (IRFs). The signals from all these pathways converge in the nucleus to produce regulated changes in the cells' transcriptional profiles. However, transcription factors are not the only molecules regulating gene expression. In eukaryotic cells, DNA and interacting proteins form a dynamic structure called chromatin. The availability of regulatory DNA elements to transcription factors and RNA polymerase II in the chromatin context is regulated by covalent modification of DNA-associated proteins and, in some cases, the DNA itself.

Transcriptional activity of transcription factors can be modulated in several ways. One is the activation of pre-existing proteins by covalent modifications. Additional modifications may also serve to stabilize the interactions between DNA and proteins to prolong the time of transcriptional activation. The activity of a given transcription factor can be targeted to a subset of immune cells by cell type-specific expression,

enabling the formation of specific transcription factor complexes exclusively in that cell type. Another possibility is to modulate the availability of transcription factors by up- or down-regulating their expression levels. Such transcription factor cascades give the possibility to robustly regulate a second wave of target genes after inducible or constitutively expressed inhibitory proteins have terminated the initiating stimulus. All of these strategies are used by cytokines in their signal transduction in target cells and some of them are also demonstrated in this work.

In this work, cells of the human immune system, monocytes, macrophages, DCs, NK and T cells, were used as a cell model. Expression and activation of STAT and IRF family transcription factors in response to cytokine stimulation was studied in these cells. Also, the effect of differentiation of monocytes to macrophages or DCs on STAT and IRF expression and activity was characterized. Interferon (IFN) stimulation increased the expression of signalling components essential for IFN responses, Stat1, Stat2, and IRF9, thus providing a mechanism for positive feedback in the IFN system. Differentiation of monocytes into macrophages was found to significantly alter the responsiveness of the cells to the growth-promoting cytokine granulocyte/macrophage colony-stimulating factor (GM-CSF), leading to significantly reduced activation of Stat5 and decreased expression of its target genes in macrophages. In NK and T cells, cytokines induced the expression of IRF1, IRF4, and IRF8 via STAT activation. IRF4 and Stat4 transcription factors were constitutively expressed only in DCs but not in macrophages.

IRFs and STATs bind to distinct DNA elements. They have both common and unique target genes. Activation of the JAK–STAT pathway in response to cytokine stimulation is usually short-lived. When IRF expression in the cell is up-regulated by STATs, the expression of target genes common to both STATs and IRFs can be regulated even though the initiating cytokine signal has already disappeared. Also, a second wave of cytokine target gene expression can be initiated by IRFs. The interplay between STATs and IRFs thus enables the cell to prolong and fine-tune the effect of the cytokine signal it has received.

2 REVIEW OF THE LITERATURE

2.1 Cytokines

In multicellular organisms intercellular communication is essential for the maintenance of homeostasis. Direct cell–cell contacts, contacts with the extracellular matrix, and signals delivered by soluble mediators provide the cell with information from the extracellular environment. This information is then integrated at the cellular level and interpreted into changes in the transcriptional profile of the cell. This ultimately leads to decisions concerning cell fate, e.g. growth, differentiation, and apoptosis.

Cytokines are soluble mediators that usually operate at very low concentrations in auto- or paracrine fashion. One exception is the diffusible chemotactic gradient created by chemokines, where local concentrations of the chemokine can reach high levels. Many different cell types can produce a given cytokine. The cytokine system displays two typical features: redundancy and pleiotropy. Two individual cytokines can be structurally very distantly related, but may nevertheless have very similar, or redundant, effects on their target cells. On the other hand, a particular cytokine may have a range of different target cells, where it regulates distinct cell type-specific processes, referred to as pleiotropy. Cytokine function in the target cell can be modulated by other cytokines or mediators (e.g. prostaglandins) simultaneously present. Often stimulation with two cytokines results in synergistic potentiation of the cytokine signals, but two opposing cytokines can also antagonize each other's effects on the target cell. A cytokine can also induce or inhibit the production of other cytokines, leading to the generation of cytokine cascades. A key determinant in cytokine responses is the expression of a suitable cell surface receptor. Receptor expression may be regulated developmentally or induced by cytokines or cell–cell contact. The cytokine–receptor interaction can be further modulated by expressing soluble or non-signaling decoy receptors, or receptor antagonists that are able to inhibit cytokine binding and subsequent intracellular signaling initiated by the receptor [1, 2].

Cytokines function as intermediates in controlling cell growth and differentiation and in regulating inflammatory reactions and the activation of antimicrobial defence systems. Classification of cytokines into families based on their structure is not very informative since cytokines are structurally very heterogenous. More traditionally, cytokines have been classified into families broadly based on their functions (Table 1), or alternatively, by the structure of their receptors (Table 2) [1, 2].

Table 1. ***Classical cytokine families***

Cytokine family	Cytokines	Biological activities
Interleukins (ILs)	IL-1–IL-31	numerous overlapping and nonredundant activities, e.g. regulation of cell growth and differentiation, proinflammatory effects, immune cell activation
Interferons (IFNs)	IFN-α, -β, -γ, -λ, -ω, -τ	antiviral, antiproliferative, immunomodulation
Tumor necrosis factors (TNFs)	TNF-α, TNF-β	proinflammatory, apoptosis
Transforming growth factor (TGF)-β family	TGF-βs, inhibins/activins, bone morphogenetic proteins	morphogenesis, development, immunomodulation
Chemokines	chemokines (C, CC, CXC, and CX_3C types)	chemotaxis, immune cell activation
Colony stimulating factors (CSFs)	GM-CSF, G-CSF, M-CSF, IL-3	growth and differentiation of hematopoietic cells
Growth factors	EPO, TPO, EGF, FGF, PDGF	growth and differentiation of cells

Adapted from [1, 2].

As cytokines are potent activators of diverse cellular processes, it is no surprise that their expression is controlled at multiple levels. Transcription of most cytokine genes requires the concerted action of several transcription factors, activated by diverse ligands, to certify that the incoming signals are properly integrated. The stability of cytokine mRNAs is also regulated, as many of them contain signals for rapid degradation in the 3' untranslated regions. Some cytokines (e. g. IL-1β and IL-18) require posttranslational processing to attain functional maturity. In addition, the secretion of mature cytokines can be a regulated process. Also, cytokines (e.g. TGF-

β) can interact with the extracellular matrix to regulate their availability to target cells after secretion.

Table 2. ***Classification of cytokines based on receptor subclass***

Cytokine receptor family	Receptor subtype	Ligands
Type I (hematopoietin)	Cytokines sharing β_c	IL-3, IL-5, GM-CSF
	Cytokines sharing γ_c	IL-2, IL-4, IL-7, IL-9, IL-13, IL-15, IL-21
	Cytokines sharing gp130 or a related protein	IL-6, IL-11, IL-12, IL-23, IL-27, LIF, OSM, CNTF, G-CSF
	Single-chain receptors	GH, EPO, PRL, TPO
Type II (IFN)	-	IFN-α/β, IFN-γ, IL-10 family
Type III (TNF)	-	TNF-α, TNF-β
Type IV (IL-1)	-	IL-1 family, IL-18
Receptor tyrosine kinase	-	M-CSF, PDGF, EGF
Chemokine	CXC receptors	CXC chemokines
	CC receptors	CC chemokines
TGF-β	-	TGF-β family

Adapted from [1].

2.2 Cytokine signal transduction pathways

2.2.1 Receptor systems utilized by cytokines

In contrast to structurally divergent cytokines, cytokine receptors can be grouped into families based on conserved structural features (Table 2). The type I (also called the hematopoietin receptor family) and type II (IFN receptor family) cytokine receptor families are structurally closely related. Receptors belonging to these families do not contain enzymatic activity, but are instead associated with cytoplasmic kinases capable of transmitting the extracellular signal inside the cell. Many type I and all type II receptors are composed of multiple subunits. In the case

of type I receptors, one of the subunits (or even two in the case of IL-2 and IL-15) can be shared between different receptor complexes. Three subgroups, sharing signal transducing chains β_c, γ_c, or gp130, can be distinguished. Some receptors belonging to the type I receptor family consist of only one type of subunit (single-chain receptors), but the active receptor is a homodimer or homo-oligomer. Type III or TNF-family receptors form homotrimers, to recognize their trimeric ligands. Instead of activating cytoplasmic kinases, these receptors signal by homotypic protein–protein interactions. Some growth factors utilize receptors containing inherent tyrosine kinase activity. Ligand binding to these receptors leads to receptor chain dimerization and activation of the kinase domains through conformational changes. Chemokine receptors belong to the seven-transmembrane domain receptor family utilizing trimeric G-proteins in intracellular signaling. TGF-β receptors, in turn, contain serine/threonine kinase activity [1-4].

2.2.2 The JAK–STAT pathway

Transduction of cytokine signals from the type I and type II cytokine cell surface receptors to the nucleus is mediated by the JAK–STAT pathway (reviewed in [5, 6]; Fig. 1). This pathway was first identified in the context of IFN signaling (reviewed in [7]). JAK tyrosine kinases associate with the cytoplasmic domains of cytokine receptor chains. Cytokine binding leads to homo- or heterodimerization, or in some cases heterotrimerization, of the receptor chains. According to the present model, these events trigger the activation of the JAK kinases [8, 9]. For the EPO receptor it has been demonstrated that in addition to receptor dimerization, conformational changes in the receptor are required for Jak2 activation [10, 11]. Juxtaposed JAKs auto- or transphosphorylate each other and specific tyrosine residues on the receptor chains. This creates docking sites for proteins containing the phosphotyrosine-binding SH2-domain. One group of such proteins is the STAT family. STAT proteins dock onto their target cytokine receptors and become phosphorylated on specific C-terminal tyrosine residues by JAKs. Some STATs require additional serine phosphorylation for full transcriptional competence (reviewed in [12]). Phospho-STATs disengage from the receptor to form homo- or heterodimers, and are then transported to the nucleus, where they bind to their target DNA sequence, the GAS element (consensus sequence $TTCN_{2\text{-}4}GAA$) [13].

The classical STAT activation pathway described above is associated with cytokine stimulation. STAT activation is usually rapid and transient in nature and is quickly down-modulated to keep cytokine responses under control. More recently, Stat1 has also been found to participate in the regulation of constitutive gene expression. Basal expression levels of certain caspase genes and the gene coding for low

molecular mass polypeptide 2 are lower in Stat1-null cells compared to the parental cell line. Basal expression is rescued to normal levels by reintroducing Stat1 or a mutant form of Stat1 that cannot be tyrosine-phosphorylated and cannot therefore form dimers. This suggests that Stat1 can also function in a non-tyrosine-phosphorylated form in target gene regulation [14, 15].

The JAK–STAT pathway is negatively regulated by several different mechanisms (reviewed in [16, 17]). The cytokine receptor can be endocytosed and lysosomally degraded, terminating the initiating signal. Protein tyrosine phosphatases, like CD45 and SHP-1, are responsible for dephosphorylation of the JAKs and the receptor phosphotyrosines. STAT DNA-binding can be down-regulated by tyrosine dephosphorylation and degradation [16, 18]. More specific mechanisms inhibiting STAT activity include the SOCS and PIAS family proteins (reviewed in [17, 19-21]).

The SH2-domain-containing, cytokine-inducible SOCS proteins bind to phosphotyrosines in cytokine receptors or JAKs, and inhibit JAK activity and STAT activation by blocking the required docking sites [19, 20]. Additionally, SOCS proteins function as ubiquitin E3 ligases, thereby mediating proteasomal degradation of interacting proteins, like the STATs. Constitutively expressed PIAS proteins target activated STATs to prevent their DNA-binding and/or transcriptional activation (reviewed in [17, 21]). Recently, PIAS proteins have been found to function in an analogous manner as SUMO-1 ligases for STAT proteins (reviewed in [22, 23]), but the importance of this feature in regulating cytokine signaling still remains to be elucidated. SUMOylation, unlike ubiquitination, does not target proteins for proteasomal degradation. SUMOylation may function as a marker sequestering activated STAT dimers and other transcription factors into specific nuclear compartments, and in this way have an impact on regulation of transcription [21, 24, 25].

Several cytokines promote the growth of their target cells, and the JAK–STAT pathway is also involved in cell growth regulation (reviewed in [26]). Inappropriate activation of STATs (especially Stat1, Stat3, and Stat5) occurs in primary human tumor cells as well as in transformed cell lines (reviewed in [27-29]), and STATs regulate the expression of many genes controlling cell growth. In human acute lymphoblastic leukemia, the Jak2 kinase domain is involved in a chromosomal translocation that creates a constitutively active Jak2–Stat5 pathway, directly contributing to the malignant phenotype [30-32]. Also, epigenetic silencing of SOCS expression may be responsible for constitutive activation of the JAK–STAT pathway [33]. Some cytokines, like IFNs, also have antiproliferative effects, but neither cellular transformation nor development of leukemia occurs in the gene knock-out mouse models of this pathway (Table 3 and 4).

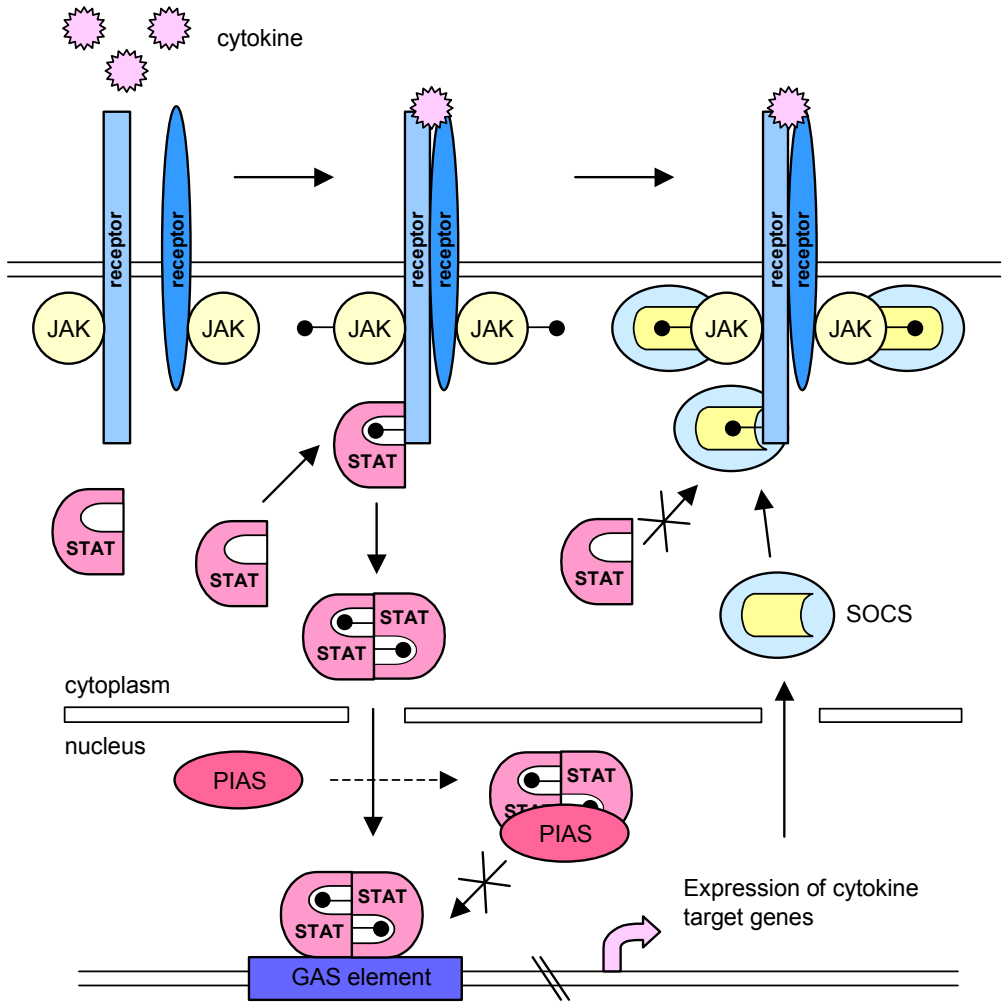

Figure 1. ***Regulation of the JAK–STAT pathway by cytokine stimulation.*** Cytokine stimulation leads to dimerization of the receptor chains. This juxtaposes the receptor-associated JAK kinases leading to their activation and tyrosine phosphorylation of the receptor chains and JAKs. Cytoplasmic STATs are recruited to specific phosphotyrosines in the receptor and are also tyrosine-phosphorylated. Phosphorylated STATs disengage from the receptor and form dimers via phosphotyrosine–SH2-domain interactions. STAT dimers are actively transported into the nucleus where they bind to GAS elements in the promoters of cytokine-regulated target genes. SOCS family proteins are cytokine-inducible, and function in down-modulating STAT responses by binding to activated JAKs and receptor phosphotyrosines preventing STAT activation. Constitutively expressed PIAS proteins also inhibit STAT DNA-binding activity by direct protein–protein interactions.

J A K s

Four different JAK kinases are found in mammals, namely Jak1, Jak2, Jak3, and Tyk2 [34-41]. They are ubiquitously expressed, with the exception of Jak3 that is only expressed in hematopoietic cells. A given JAK kinase can be activated by a variety of cytokine receptors (Table 3). The structure of the JAKs is unusual as they contain, in addition to a functional kinase domain, a catalytically inactive pseudokinase domain [5]. This domain regulates both the basal and cytokine-induced activity of JAKs [42-44]. Also, JAKs lack SH2- and SH3-domains, protein-protein interaction domains typical for cytoplasmic tyrosine kinases.

Mice with targeted disruptions of the JAK genes have been generated (Table 3) [45-52]. Gene deficiencies reveal some characteristic signaling defects, but as targeting of Jak1 and Jak2 results in perinatal or embryonic lethality, tissue-specific gene-targeting studies could yield more information on JAK functions.

Table 3. ***Activation of JAKs by cytokines and phenotypes of JAK-deficient mice***

Activating cytokines	**JAK**	**Phenotype of knock-out mice**
IFNs, IL-10, IL-6 family cytokines, IL-2, IL-4, IL-7, IL-9, IL-13, IL-15, G-CSF, IL-3, IL-5, GM-CSF	**Jak1**	Perinatal lethality. No responses to IFNs, IL-2, IL-4, IL-7, IL-9, IL-10, IL-13, IL-15. Reduced responses to IL-6 and LIF.
IFN-γ, IL-6 family cytokines, G-CSF, IL-3, IL-5, GM-CSF, PRL, EPO, TPO, GH	**Jak2**	Embryonic lethality. Lack of definitive erythropoiesis. No responses to IFN-γ, IL-3, IL-5.
IL-2, IL-4, IL-7	**Jak3**	Severe combined immunodeficiency.
IFN-α/β, IL-6 family cytokines, IL-10	**Tyk2**	Subtle defects in IFN-α/β- and IL-10-signaling, defective responses to IL-12 and LPS.

Adapted from [9, 16, 53].

S T A T s

The STAT family of transcription factors comprises seven members: Stats 1-4, Stat5A and Stat5B, and Stat6 [54-66]. STATs contain a homologous DNA-binding domain, an SH2-domain, and a conserved tyrosine that is phosphorylated by JAKs in response to receptor activation (reviewed in [5, 67, 68]). The conserved N-terminal region is required for protein–protein interactions between STAT dimers [69, 70], and promotes cooperativity in DNA-binding. The more divergent C-terminal end contains the transcriptional activation domain. As is the case with JAKs, most STAT proteins are activated by several cytokines, notable exceptions being Stat4 and Stat6 that are activated almost exclusively by IL-12 and IL-4, respectively [71]. However, generation of STAT-deficient mice has revealed the biological specificity of STAT activation (Table 4; reviewed in [68, 72]; [73-84]). Additional cytokine target-gene specificity arises from subtle differences in the affinities of different STAT dimers to variations in the GAS consensus sequence [13]. All STATs except Stat2 homodimerize, which is the most typical dimer form, but heterodimerization occurs between Stat1 and Stat2, Stat1 and Stat3, and Stat5A and Stat5B [5].

Table 4. ***Activation of STATs by cytokines and phenotypes of STAT-deficient mice***

Activating cytokines	**STAT**	**Phenotype of knock-out mice**
IFNs IL-6 family cytokines	**Stat1**	Impaired IFN signaling and antiviral responses.
IFN-α/β	**Stat2**	Impaired type I IFN-dependent immune responses.
IL-6 family cytokines, G-CSF, IL-2, IL-10	**Stat3**	Embryonic lethality.
IL-12, IFN-α	**Stat4**	Impaired responses to IL-12 (defective Th1 development).
IL-2, IL-7, IL-9, IL-15, IL-3, IL-5, GM-CSF, PRL, GH, EPO	**Stat5A**	Impaired mammary development, partial T cell growth defect.
	Stat5B	Loss of sexually dimorphic growth in males. Defects in T cell growth, NK cell development, and GM-CSF signaling in macrophages.
IL-4, IL-13	**Stat6**	Impaired responses to IL-4 (defective Th2 development).

Adapted from [16, 68, 72].

All STAT proteins, with the exception of Stat2, also exist as smaller molecular weight variants, referred to as β-isoforms as opposed to full-length α-isoforms. The expression patterns of the β-variants differ from ubiquitous to cell type-restricted expression, and they originate from alternatively spliced mRNAs, or are posttranslationally processed by proteolysis [85, 86]. The truncation is C-terminal involving the STAT transcriptional activation domain. Alternatively spliced Stat1β supports IFN-α-, but not IFN-γ-induced transcription [87] due to the lack of the essential serine phosphorylation site. Alternatively spliced Stat3β, together with c-jun, activates transcription from a composite element [88], and has distinct roles from those of Stat3α [89]. By microarray analyses, Stat4α and Stat4β were found to induce partly overlapping but partly unique target genes [90]. Expression of smaller forms of Stat5 is detected in hematopoietic progenitor cells [63, 91], and their expression is gradually lost during terminal differentiation [92, 93]. The expression of Stat6β in murine mast cells limits the effect of IL-4-stimulation in these cells [94, 95]. Originally, the truncated forms were assigned an exclusively dominant negative role in transcriptional regulation [93, 96-99], but increasing evidence supports their activity as bona fide transcription factors with their own target gene specificities [89, 90].

In addition to forming STAT dimers, STATs interact with other transcription factors. At least members of the IRF family, NF-κB, Sp1, c-jun, and the glucocorticoid receptor are known to regulate transcription from adjacent or composite DNA-elements (reviewed in [85, 100]). Also, direct interactions between STATs and transcriptional coactivators are detected [85, 100].

2.2.3 IRFs

The IRF family has nine members in mammals, of which only IRF6 remains functionally uncharacterized (Table 5; reviewed in [101, 102], [103-118]). Additional IRF-like proteins are encoded in the genome of human herpes virus-8 [119]. The members of the IRF family share a homologous DNA-binding domain that binds to variations of a direct tandem repeat element termed IRF-E (consensus sequence $GAAA^{G}/_{C}{}^{T}/_{C}GAAA^{G}/_{C}{}^{T}/_{C}$) [101, 102]. Viral IRF-homologs interfere with the functions of cellular IRFs [120, 121] and inhibit transcriptional activation, thereby inhibiting antiviral and growth-suppressive activities of IFNs [122]. IRF expression patterns and effects of IRF gene targeting in mice are summarized in Table 5.

The IRF family can be divided into two subfamilies on the basis of participation in the IFN response to viruses. IRF3, IRF5, IRF7, and IRF9 are essential for induction of IFN-α/β gene expression in response to viral challenge [112, 122-125]. IRF1 and

IRF2 also participate in, but are not essential for, the induction of IFN gene expression [126]. IRF1 regulates the expression of genes belonging to the IFN system, like 2', 5'-oligoadenylate synthetase and dsRNA-dependent protein kinase. Additionally, IRF1 controls the expression of several other genes involved in innate and adaptive immune responses, like $gp91^{phox}$, IL-12/p40, IL-15, and genes needed for MHC class II expression (reviewed in [127]). Expression of IRF4 and IRF8 is limited to lymphoid and myeloid cells (Table 5), and they are strongly implicated in controlling cellular differentiation and homeostasis of these cell types, as well as several aspects of immune responses [128-137].

Table 5. ***The expression of IRFs and effect of gene deficiency***

IRF	Expression	Phenotype of the immune system in knock-out mice
IRF1	ubiquitous, strongly cytokine-inducible	impaired NK cell development, defective Th1 responses, defective CD8+ T cell development
IRF2	ubiquitous	inherently impaired NK cell development and activity, dysregulation of IFN-α/β-responses
IRF3	ubiquitous	defective antiviral response
IRF4	myeloid and lymphoid cells	defective CTL and antitumor responses, defects in B cell development and antibody production
IRF5	lymphoid tissue, peripheral blood lymphocytes	-
IRF6	only cDNA sequence data available	-
IRF7	ubiquitous; IFN-α/β-inducible	defective antiviral response
IRF8	myeloid and lymphoid cells	defective Th1 responses (lack of IL-12), CML-like syndrome
IRF9	ubiquitous	defective antiviral response
vIRFs	human herpes virus 8	-

Summarized from [101, 102].

In the original studies IRF1 and IRF9 (in the context of ISGF3) were described as transcriptional activators, whereas IRF2, IRF4, and IRF8 were denoted as repressors [101, 102]. However, the repressor function seems to depend on promoter DNA elements and interacting transcription factors. To date, IRF2, IRF4, IRF5, and IRF8 have been found to either activate or repress gene expression, depending on the target gene (reviewed in [101, 102, 138, 139]). IRFs form several inter-family homo- or heterodimers. In addition, IRFs have been found to interact with basal transcription machinery proteins [140-143] as well as several other transcription factor family members [144-149].

Several IRFs are posttranslationally modified by phosphorylation on serine residues [101]. This modification affects their activity, subcellular localization, and ability to interact with other IRFs and other transcription factors. Kinases reported to induce serine phosphorylation of various IRFs in biochemical assays include casein kinase 2, protein kinase A, protein kinase C, IKKε, and TANK-binding kinase-1 [101, 150, 151]. Several conserved tyrosine residues are found in the IRF DNA-binding domain, but so far tyrosine phosphorylation has been found to regulate the DNA-binding of IRF8 only [152].

Initially, IRF1 and IRF2 were described as having antioncogenic and oncogenic potentials, respectively, when overexpressed [153, 154]. IRF1 regulates the cell cycle [155] and DNA damage-dependent apoptosis [156]. In the human genome, the IRF1 gene maps to the chromosomal region 5q31.1 [157]. Deletions encompassing this region are frequently found in patients with leukemia or preleukemic myelodysplastic syndrome. Expression of IRF4 in human T cells is induced by the Tax oncoprotein of HTLV-1 [111]. Also, a chromosomal translocation involving the IRF4/multiple myeloma-1 and Ig heavy-chain loci is frequently found in patients with multiple myeloma [158]. Mice with disrupted IRF8 gene develop a human CML-like syndrome [131]. Altered expression of IRF8 is likely to be involved in the pathogenesis of the human disease as well, as CML-patients express only very low numbers, if any, of IRF8 transcripts [159]. IFN-α is used as a therapeutic agent in the treatment of CML, and an increase in IRF8 expression correlates with a favorable cytogenetic response to IFN-treatment [160].

2.2.4 NF-κB

The mammalian NF-κB/Rel family has five members, NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB, and c-Rel. They all contain an N-terminal Rel homology domain that mediates NF-κB homo- or heterodimerization, DNA-binding, and binding to inhibitory IκB-proteins (reviewed in [161-163]). The Rel homology domain also contains the nuclear localization signal. NF-κB dimers interact with

inhibitory IκB proteins in the cytoplasm [161-163]. NF-κB1 and NF-κB2 are synthesized as precursor proteins of 105 kDa and 100 kDa, respectively. These precursor proteins contain internal IκB-like regions and require proteolytic processing to generate the active form. IκB interaction prevents nuclear translocation of NF-κB dimers by masking the nuclear localization signal. Cellular stimulation activates the classical IKKα/β/γ complex that phosphorylates the IκB proteins and targets them for proteasomal degradation, thereby enabling active NF-κB dimers to enter the nucleus. The NF-κB response is regulated by a negative feed-back loop: NF-κB dimers activate the expression of IκB that is able to terminate the NF-κB response by binding to NF-κB dimers and causing their dissociation from the DNA (reviewed in [161, 164]). Proteasomal degradation of activated NF-κB dimers is also involved in regulating the length of the NF-κB response [165].

NF-κB functions as a central regulator of the cellular stress response. It is activated by inflammatory cytokines (e.g. TNF-α, IL-1, IL-18), growth factors, bacteria and viruses, UV irradiation, certain therapeutic agents, complement, and oxidative stress, among others [161, 164]. NF-κB signaling pathway is involved in the control of the cell cycle, apoptosis, lymphocyte development and maturation, as well as neuronal cell function [161, 166, 167]. Target genes activated by NF-κB include adhesion molecules, cell-surface receptors, chemokines, ILs, and growth factors. Specificity in NF-κB target gene regulation is achieved through several mechanisms. Not all NF-κB dimers are formed with equal efficiency (Table 6), and the RelA(p65)- and NF-κB1(p50)-homodimers and p65/p50-heterodimers are the most common ones [162, 168]. Individual dimers are differentially activated in response to diverse stimuli, and show preference in binding to different subsets of target sites (reviewed in [168, 169]). NF-κB1 and NF-κB2 lack a transactivation domain, and homodimers of them are transcriptionally repressive. Some NF-κB proteins show cell type-restricted expression patterns (Table 6), conferring additional specificity of signaling [170]. Also, several IκB isoforms exist with distinct affinities towards different NF-κB dimers [164]. Interestingly, distinct NF-κB dimers regulate the immediate and sustained NF-κB-induced transcriptional activation during DC maturation, and a temporally regulated exchange of NF-κB dimers takes place at several target promoters [171].

Recently, an alternative NF-κB pathway involving NF-κB2/p100 processing by nuclear factor-inducing kinase (NIK)/IKKα was identified [172]. It is activated with slower activation kinetics than the canonical pathway by a group of receptors belonging to the TNF receptor family. This pathway is essential for lymphoid organ development and adaptive immunity. Especially important is the activation of RelB/NF-κB(p52) dimers by the lymphotoxin β -receptor [173]. Splenic architecture

and B cell functions are affected in mice with deletions in genes coding for the activators and signaling intermediates of this pathway [162, 163].

Table 6. ***Expression patterns of NF-κB proteins and possible dimer combinations***

NF-κB protein	Expression pattern	Dimerization partners
NF-κB1(p50)	ubiquitous	RelA(p65), RelB, c-Rel, NF-κB1(p50)
NF-κB2(p52)	ubiquitous	RelA(p65), RelB, c-Rel, NF-κB2(p52)
RelA(p65)	ubiquitous	RelA(p65), c-Rel, NF-κB1(p50), NF-κB2(p52)
RelB	hematopoietic cells	NF-κB1(p50), NF-κB2(p52)
c-Rel	hematopoietic cells	RelA(p65), c-Rel, NF-κB1(p50), NF-κB2(p52)

2.2.5 The MAPK pathway

The MAPK family consists of four related subfamilies: the ERKs, the JNKs, p38, and ERK5 (reviewed in [174, 175]). The MAPKs themselves are serine/threonine kinases that phosphorylate direct targets in the cytoplasm or inside the nucleus. Additionally, they phosphorylate and activate various downstream protein kinases [175]. MAPKs are activated by MAPK kinases (MAPKKs), a family of dual-specificity kinases that activate the MAPKs by both threonine and tyrosine phosphorylation. MAPKKs are activated by serine/threonine phosphorylation by upstream MAPK kinase kinases (MAPKKKs). The signaling pathways activating MAPK cascades include Ras/Raf, Ras/PI3K, and Cdc42/Rac pathways, and receptor-coupled trimeric G-protein signaling [174, 176]. IFN-α/β can activate the p38 MAP kinase [177].

MAPK signaling pathways are activated by inflammatory cytokines, growth factors, and cell stress, resulting in changes in the expression levels or intercellular localization, altered DNA-binding or transactivation capacity of various transcription factors [175]. These transcription factors then activate the expression of genes involved in inflammation, apoptosis, regulation of growth, and cellular differentiation. The ERK and JNK kinases have been linked to the regulation of the JAK–STAT pathway as potential kinases responsible for C-terminal STAT serine phosphorylation. Apparently, serine phosphorylation of a given STAT can be mediated by various MAPK pathway kinases, depending on the signaling pathway

involved (reviewed in [12, 177]). Also, signals from the same receptor can enhance the serine phosphorylation of different STATs via distinct pathways [12].

One target of the MAPK pathway is the Ets transcription factor family. The Ets family consists of approximately 30 members in mammals, and includes both transcriptional activators and repressors (reviewed in [178, 179]). Ets transcription factors control the expression of many receptors for hematopoietic growth factors and cytokines, like GM-CSF, M-CSF, G-CSF, and IL-2. Several Ets factors interact with STATs and IRFs, among others, in target gene regulation [179, 180].

The MAPK pathways also influence the activity of the AP-1 complex, a dimeric transcription factor formed by members of the Jun/Fos family of transcription factors. MAPKs act both by increasing the expression and stability of AP-1 components and by directly stimulating AP-1 activity (reviewed in [181, 182]). In addition to the JAK–STAT pathway and Ets and AP-1 transcription factors, the MAPK pathway regulates the activity of several additional transcription factors (reviewed in [175]).

2.3 Chromatin and transcriptional control

Eukaryotic DNA in the nucleus is packaged into chromatin by association with specific proteins. The chromatin is a highly dynamic structure where densely packed, transcriptionally silent areas exist alongside more relaxed, open areas of actively transcribed genes. Also other cellular processes like DNA replication and mitosis affect the packaging ratio of the chromatin. The basic unit of chromatin is the nucleosome, where the DNA is wrapped twice around a unit of eight histone molecules. Approximately 150 bp of DNA is associated with the core histones (comprising dimers of histones H2A, H2B, H3, and H4). Histone H1 is located outside the core particle, presumably interacting with the linker DNA between adjacent nucleosomes [176]. There are variants of histones that affect the structure and functionality of the nucleosome (reviewed in [183]), like the H2A.Z variant that is involved in preventing the spread of heterochromatin into euchromatic chromosomal areas [184], and H3.3 that it a marker for transcriptionally active regions in Drosophila [185]. The nucleosome fiber is further packed into poorly characterized higher-order structures together with other structural proteins.

To assist the interaction between promoter DNA elements and regulatory transcription factors, and to enable transcription to initiate and proceed, the barrier formed by the chromatin structure must be circumvented. Two main classes of enzymes are used by the cell in this process, the ATP-dependent chromatin remodeling complexes (e.g. SWI/SNF complex) and enzymes able to covalently

modify histones (e.g. histone acetyl transferases, or HATs) (reviewed in [186, 187]). These regulators seem to act in a synergistic manner to alter the chromatin structure, but their order of action is still somewhat controversial and may differ depending on the cellular process or the target promoter [186, 187]. For example, in the late phases of yeast mitosis, chromatin remodeling is a prerequisite for HAT recruitment [188, 189], but in transcriptional induction of the human IFN-β gene, HAT activity is essential for the recruitment of SWI/SNF-like remodeling enzymes [190]. There is evidence that transcription factors and gene promoters show specific requirements in recruiting transcriptional coactivators with HAT activity [191].

2.3.1 Chromatin remodeling enzymes

The chromatin remodeling complexes are large aggregates of proteins. They use the energy derived from ATP-hydrolysis to cause reorganization of the nucleosomal structure in a non-covalent manner (reviewed in [192]). Three main groups of such complexes are ISWI, Mi-2, and SWI/SNF families of chromatin remodelers [186, 192]. Function of ISWI and Mi-2 complexes is mainly associated with transcriptional repression, whereas SWI/SNF complexes are activators. Possible mechanisms leading to nucleosome remodeling include sliding of histone octamers, transfer of histones to another DNA molecule, and induction of conformational changes in either histones, DNA, or both [186]. Nucleosomal sliding does not increase the amount of exposed DNA, but only changes its localization. Different complexes may use different remodeling mechanisms, depending e.g. on the nucleosomal density of the target chromatin area [186].

Two models have been proposed for the action of the SWI/SNF enzyme complex. In both models, a twist in DNA is formed in an ATP-dependent manner. This twist then creates a wave of accessible DNA on the histone octamer. In the first model, the SWI/SNF complex tracks along the DNA, simultaneously inducing torsional stress. The second model proposes that the twist is created by continuously inducing helix deformation. In both cases, the action of the SWI/SNF enzymes only disrupts the DNA–histone interface, leaving the histones in place and histone–histone interactions intact [187]. Both transcriptional activators and general transcription machinery may be able to recruit the SWI/SNF complex to specific promoters [193].

2.3.2 Histone modifying enzymes

Histones are highly basic proteins with a globular structure. However, the amino-terminal tails of histones protrude from the nucleosome, and specific lysine, arginine, and serine residues are available for covalent modification. Histones are

targets for several different chemical modifications: acetylation, methylation, phosphorylation, ubiquitination, ADP-ribosylation, and glycosylation (reviewed in [194, 195]). These modifications can modulate the DNA-packing ability of the histones by decreasing their net positive charge. Alternatively or additionally, they may create docking sites for interacting proteins [194]. Accordingly, many transcriptional coactivators, i.e. activator proteins that do not directly bind to DNA but bind to DNA-binding proteins instead, contain bromodomains and chromodomains, protein domains recognizing acetylated and methylated lysine residues, respectively [195].

HATs are the enzymes responsible for acetylation of histones, and their action is opposed by histone deacetylases (HDACs). Acetylation of histones can destabilize higher-order chromatin structures [187], and amino-terminal histone acetylation is linked to transcriptional activity of a given gene. This modification is reversible, and deacetylation is known to repress transcription. Many transcriptional coactivators contain inherent HAT activity. In addition to acetylating histones, these enzymes target also non-histone proteins, like sequence-specific transcription factors.

Histone methylation has several consequences, such as gene activation or repression, or heterochromatic gene silencing, depending on whether the target residue is an arginine or a lysine [194-196]. Furthermore, a lysine can be mono-, di- or trimethylated [195, 197]. Methylation of histones is considered a stable epigenetic mark [198], as no histone demethylases have been identified. However, nucleosomal histone methylation status can be altered and involves histone exchange [198].

Histone phosphorylation may regulate protein interaction events by creating a "charge patch" rich in negatively charged residues [195]. Phosphorylation of the linker histone H1 leads to increased dissociation from the chromatin in *Tetrahymena* [199]. Histone acetylation and phosphorylation are also implicated in repairing double-stranded breaks in the DNA [195].

The histone code

In the year 2000, B.D. Strahl and C.D. Allis coined the "histone code" hypothesis [200]. This hypothesis maintains that a particular biological outcome is dictated by the sum of histone modifications present in the nucleosomes. The association of additional proteins with chromatin is controlled by the specific histone modification patterns, and this association leads to changes in the functional state of the DNA involved. This model necessitates the existence of proteins able to create the histone code (histone modifying enzymes), and proteins able to decipher it (bromodomain- and chromodomain-containing proteins). Strict histone modification patterns can be detected in various chromatin regions, like actively transcribed areas or transcriptionally silent regions. Recently, the histone acetylation code for the IFN-β-

promoter has been resolved [201], supporting the histone code hypothesis. The sequential order in which these modifications take place is significant, and some modifications are a prerequisite for, or prevent, a subsequent modification. This type of regulation can also be extended *in trans* from one histone tail to another [202, 203]. All in all, recent evidence supports the concept of the histone code, but at the same time underlines the complexity of this regulatory mechanism.

2.3.3 The enhanceosome concept

The promoters and enhancers controlling eukaryotic gene expression consist of several short DNA elements able to recruit and interact with sequence-specific transcription factors. These modules bestow the regulatory regions with the ability to integrate various signals and to form protein surfaces able to recruit chromatin remodeling complexes and histone modifying enzymes. The paradigm of such control is the virus-inducible IFN-β enhancer (reviewed in [187, 204]). This enhancer is located between two positioned nucleosomes, and contains the binding sites for NF-κB, IRF, and ATF/c-Jun proteins. Transcriptional activation of the IFN-β gene requires that all these specific factors are present together with the HMG I(Y) protein. The role of the HMG I(Y) protein is to produce structural alterations in the DNA to enable the formation of a stable protein–DNA complex termed the IFN-β enhanceosome. The enhanceosome complex then recruits, in highly regulated sequential order, distinct coactivators with HAT activity, the chromatin remodeling complex SWI/SNF, and basal transcription machinery proteins, including RNA polymerase II, leading to nucleosome remodeling and initiation of transcription [190]. The T cell receptor α chain enhanceosome, which is developmentally regulated, and the TNF-α enhancer, which hosts a collection of binding sites used selectively by different stimuli, are examples of other well-characterized enhanceosome structures [204].

2.3.4 Transcriptional coactivators in cytokine signaling

Global transcriptional coactivators containing HAT activity, like proteins belonging to CBP/p300, PCAF/GCN5, TAF250, and SRC1 families, are involved in histone acetylation and transcriptional coactivation together with a plethora of different sequence-specific transcription factors (reviewed in [205]). Signaling proteins mediating cytokine signal transduction make no exception: STATs, IRFs, and NF-κB require various coactivator-HATs as partners in target-gene regulation.

Stat1 [191, 206, 207], Stat2 [208], and most likely also Stat4 [209, 210] interact with the CBP/p300 HATs. Stat3, Stat5 and Stat6 interact with both CBP/p300 and SRC

coactivators to obtain full transcriptional activity [211-217]. Stat5 apparently requires glycosylation of a specific residue to interact with CBP [218]. IRF1 and IRF2 interact with p300 and PCAF [141, 142]. IRF3 and IRF7, the two IRFs that are essential for type I IFN gene induction, show an interesting difference in their ability to interact with coactivators. IRF3, but not IRF7, binds CBP, p300, and PCAF [143]. This feature, together with slight differences in DNA-binding specificity, may form the basis for their differential function in regulating IFN-α/β gene expression. In IFN-γ-stimulated monocytes, IRF1, IRF8, and PU.1 form a complex able to recruit CBP, and this interaction is essential for the transcriptional induction of the $gp91^{phox}$ promoter [140]. Also NF-κB proteins RelA(p65) and NF-κB1(p50) interact with CBP/p300, PCAF, and SRC coactivators [219-221].

Lately it has become increasingly evident that other DNA-binding proteins in addition to histones are targeted by coactivator-HATs as well. Transcription factors acetylated by different HATs include, among others, p53, MyoD, HMG I(Y), GATA-1, E2F1, and c-Myb [205]. IRFs are also targets of the coactivators' HAT activity. PCAF acetylates IRF7 on a lysine residue located in the DNA-binding domain, and this modification inhibits the DNA-binding activity of IRF7 [222]. IRF1 and IRF2 are acetylated by p300 and PCAF [141, 142], and in the case of IRF2, this modification serves to target IRF2 to growth-regulated promoters [223]. RelA(p65) and NF-κB(p50) are also acetylated by CBP/p300 on several residues located mainly in the DNA-binding domain [224-226]. Acetylation of RelA(p65) regulates the transcriptional and DNA-binding activity, nuclear export, and IκBα-association of RelA(p65)(reviewed in [227]).

2.4 The immune system

The immune response can be divided into innate and adaptive responses. Physical barriers like the skin function as part of innate immunity by preventing pathogen entry. Phagocytic cells (monocyte/macrophages and neutrophils), NK cells, complement, and cytokines are the principal components of innate immunity. Characteristically, they exist before infection, their responses are rapidly activated, and their activation does not directly result in generation of immunological memory. Pathogen recognition in innate immunity relies on receptors encoded in the germ line, like the TLRs, NODs, CD14, scavenger receptors, and mannose-binding lectin [228-233]. These receptors recognize conserved structures common to pathogens but missing in the host itself. The receptors of antigen-specific adaptive immunity, antibodies and T cell receptors, display a vast degree of variability. They are selected on the basis of their affinity towards their ligand, and are generated by somatic gene rearrangements. Activation of adaptive immune responses is

characterized by a time lag of several days. The innate response protects the host during this period, and is a prerequisite for the successful initiation of the antigen-specific immune response.

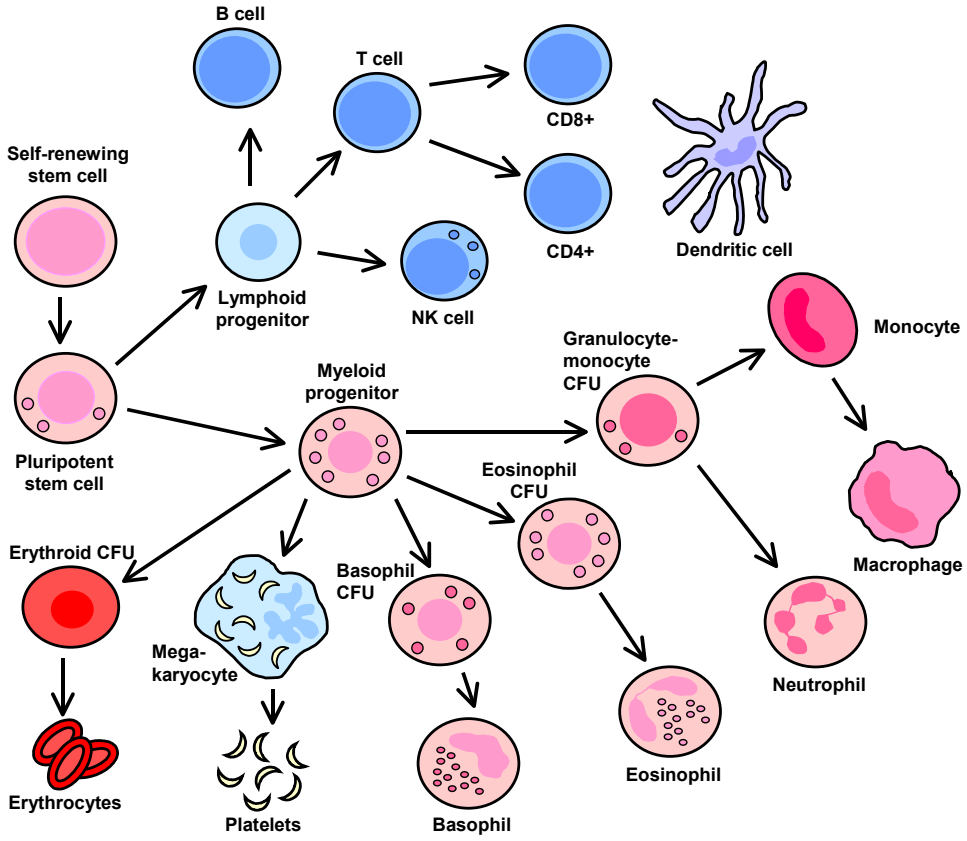

Figure 2. ***Hematopoiesis.*** The development of different lineages of blood cells schematically illustrated. Blood cells differentiate from pluripotent stem cells in the bone marrow. Bone marrow stromal cells secrete cytokines that are responsible for stimulating the proliferation and maturation of precursor cells. Dendritic cells can originate from both myeloid and lymphoid progenitors. CFU, colony-forming unit. Adapted from [234].

The shift from innate to adaptive immune responses is elicited by antigen-presenting cells (APCs). Macrophages and DCs are professional APCs that internalize extracellular antigens by phagocytosis, endocytosis, and pinocytosis, and subject them for specific intracellular processing events to create short antigen-derived peptides. These peptides are then presented in the context of MHC molecules to T cells to initiate adaptive immune responses. These cell–cell contacts between APCs and T cells and cytokines present in the cellular environment shape the development of both innate and adaptive immune responses by guiding the differentiation of immune cells and influencing their activation and functional maturation.

2.4.1 The monocyte lineage

Monocytes and monocyte-derived macrophages and DCs are a part of the myeloid cell lineage, together with erythrocytes, granulocytes, and megakaryocytes (Fig. 2). Some DCs originate also from the lymphoid lineage. Monocytes and macrophages are important mediators of innate immune responses due to their phagocytic potential and ability to produce a range of cytokines with proinflammatory, immunomodulatory, proliferative, and differentiation-inducing properties. Macrophages are also able to act as APCs, thus activating adaptive immune responses. DCs are unique APCs in the respect that they are able to activate also naive T cells, and DCs are the major cell type responsible for initiating the adaptive phase of the immune response.

Precursors for monocytes, as for other blood cells, reside in the bone marrow. Their differentiation from the pluripotent stem cells is a multi-step process guided by interactions with the bone marrow stromal cells and cytokines, mainly CSFs and various ILs (reviewed in [235-237]). Monocytes are a mature cell type with the ability to phagocytose foreign antigens and produce cytokines upon activation. Monocytes leave the bone marrow and enter the circulation. However, monocytes achieve full functional maturation only when they leave the blood stream and enter tissues, either in normal peripheral tissues or at sites of inflammation, where they are actively recruited by chemokines.

Macrophages

Macrophages are monocyte-derived tissue-resident cells that are relatively long-lived [238]. They are involved in normal homeostasis as they phagocytose apoptotic cells and participate in wound healing. Liver Küpffer cells, microglia of the central nervous system, lung alveolar macrophages, and bone osteoclasts all represent tissue-specific macrophage classes with their own specialized functional profiles [239]. A wide variety of transcription factors guides the generation of the

monocyte/macrophage population. One of the most important factors directly affecting monocyte/macrophage differentiation is the Ets-family transcription factor PU.1 (reviewed in [240]). Together with other factors, including C/EBPα and AML1, it controls the expression of the M-CSF receptor, thus making developing monocyte precursors responsive to the lineage-determining cytokine M-CSF [236, 237]. IRF8 is also important for monocyte development, as IRF8 gene targeted mice have a severely reduced and functionally defective monocyte/macrophage population, and they display enhanced granulocyte colony formation instead [130, 131].

Macrophages are activated by various inflammatory signals, like microbial products, such as LPS, and cytokines, e.g. TNF-α and IFN-γ (reviewed in [241]). Activated macrophages migrate to sites of inflammation. These cells are capable of killing and degrading intracellular microbes. They produce inflammatory cytokines, oxygen radicals, and lipid mediators [241]. Many of these substances can have deleterious effects on the surrounding tissue. Macrophage activity can be down-modulated by immunoregulatory cytokines such as IL-10 and TGF-β.

Dendritic cells

Immature DCs in the tissues are in a constant state of vigilance, and they monitor their surroundings by macropinocytosis, receptor-mediated endocytosis, and active phagocytosis (reviewed in [242]). Following the emergence of microbial threat, immature DCs leave the peripheral tissue to migrate to the local lymph nodes with the captured antigen. Mobilization and maturation of DCs are complex processes involving loss of phagocytic potential and changes in the expression of adhesion molecules, costimulatory ligands, chemokines and chemokine receptors on the surface of the DC [242]. In the lymph node, DCs select the few CD4+ Th cells able to recognize the specific antigen. Th cells are then responsible for propagating the activating signal to other cells of the adaptive immunity. The phenotype of the DCs initiating the adaptive immune response can widely influence the outcome of the T cell response: cytokine secretion by the DC, antigen concentration and number and identity of costimulatory molecules on the DC give information on the nature and localization of the pathogen to the T cell [243, 244]. In addition to their role in bridging innate and adaptive immunity, DCs are also involved in the formation of peripheral immunological tolerance [245].

The human DC population comprises several specialized subtypes that differ in their differentiation lineage, phenotype, function, and localization in the body. Epidermal Langerhans cells, interstitial DCs, and plasmacytoid DCs are generally recognized as distinct subtypes [246]. However, there is considerable phenotypical and functional plasticity among DC subtypes, and the expression of distinct cell-surface

markers may reflect the maturation or activation status or tissue origin of DCs rather than define clear-cut subpopulations. Gene-targeting studies in mice have pointed out a role for several transcription factors, like Ikaros, RelB, PU.1, and IRF8, in generating functional DCs [134-137, 247, 248].

2.4.2 NK cells

NK cells are bone marrow-derived cells that belong to the lymphocyte lineage. They function in innate immune responses by directly lysing virus-infected and tumor cells. Additionally, they produce high quantities of cytokines, e.g. IFN-γ, TNF-α, and GM-CSF, which regulate the development of adaptive immunity. The bone marrow stromal environment is critical for the generation of NK cells. Flt3L-signaling and an intact IL-15/IL-15R-system (including IL-15, IL-15Rα, IL-2Rβ, γ_c, Jak3, and Stat5) are also essential [249]. IL-15 is also required for homeostasis of peripheral NK cells. Recent findings indicate that IL-21, a T cell-derived cytokine, regulates the transition from innate to adaptive phase of the immune response by limiting NK cell activity [250].

Activation of NK cells (expansion of the NK cell population and augmented cytolytic capacity) can be mediated by target recognition and/or cytokine stimulation (reviewed in [249]). One class of NK cell receptors are inhibitory receptors that recognize self-MHC molecules [251]. This recognition precludes NK cell activation. Stimulatory, non-MHC-restricted NK cell receptors exist but their ligands remain largely uncharacterized [251]. NK cell activity is stimulated by several cytokines produced by infected DCs and macrophages, like IFN-α/β, IL-12, IL-15, and IL-18 [249]. Upon viral infection peripheral NK cells rapidly proliferate, and a two-phase model of NK cell activation has been proposed [252]. According to this model, in the early stages of infection, NK cell proliferation is activated nonspecifically by cytokines, and at the later stages, by specific interaction of activating NK cell receptors with virus-infected target cells [252].

2.4.3 T lymphocytes

T and B lymphocytes are the effector cells of specific immune responses. They express antigen-specific cell-surface receptors, the TCR on T cells and surface Ig on B cells, that specifically recognize peptides presented by APCs. The recognition of the cognate peptide in its proper MHC-context, additional costimulatory signals provided by other receptor-ligand interactions and the presence of a proper cytokine environment triggers the activation and clonal expansion of T and B cells, and execution of their effector functions. The main function of the B cell population is to

produce antibodies of various classes. The T cell population is divided into two groups, the CD8+ CTLs and the CD4+ Th cells. CTLs lyse infected or transformed target cells. The role of Th cells is to provide stimuli that activate B cell antibody production or macrophage effector functions and enhance CTL cytotoxicity.

Immunological memory is one of the hallmarks of adaptive immunity, and both T and B cells contribute to protection from reinfection. For T cells, both CD4+ and CD8+ memory cells exist [253]. Regulatory T cells form another important T cell subset. These cells function to maintain peripheral tolerance, and control autoimmunity, allograft rejection, and inflammatory reactions [254]. Regulatory T cells function by producing cytokines that suppress immune responses, like IL-10 and TGF-β, or by direct cell-cell contacts [254, 255].

Th1 and Th2 cells

Following activation by DC antigen presentation, naive CD4+ Th cells further acquire more specialized effector phenotypes. These phenotypes can be broadly classified as promoting cellular, or Th1, and humoral, or Th2, immune responses. The Th1 type immune response is characterized by the production of the hallmark cytokine IFN-γ by Th cells, and entails coordinated activation of phagocytes, production of opsonizing antibodies, and induction of CTLs. In contrast, Th2 type cells secrete IL-4. These cells activate eosinophils, mast cells, and basophils, and, in addition, stimulate IgE production by B cells (reviewed in [256, 257]).

The generation of Th1 and Th2 phenotypes is guided by the cytokine milieu at the time of activation of naive T cells. These cytokines can also be used for *in vitro* generation of Th1 and Th2 type cells. Infected APCs usually produce IL-12, IFN-α/β, IL-18, IL-23, and IL-27, and all of these cytokines, either alone or synergistically, induce the production of IFN-γ by T cells, thus favoring Th1 responses [258]. IL-4 is the major cytokine regulating the generation of Th2 cells. Furthermore, Th1 and Th2 cytokines antagonize each other's effects, e.g. by selectively down-regulating cytokine production and receptor gene expression [256, 257, 259].

Several cytokine-regulated transcription factors are involved in determining the Th cell phenotype. Stat4 and Stat6, responsible for signaling by IL-12/IFN-α and IL-4, are required for Th1 and Th2 responses, respectively. IFN-γ induces the expression of the transcription factor T-bet, which in turn promotes the expression of IL-12R and IFN-γ, creating a positive feedback loop strengthening Th1 polarization. IL-4 is involved in the induction of the transcription factor GATA-3, which is expressed in naive CD4+ Th cells at low levels, and is strongly induced in Th2 cells [256, 257, 259].

Structural changes that persist after cell divisions are also detected in the chromatin surrounding *IL-4* and *IFN-γ* loci during Th cell differentiation. T-bet and GATA-3 have been implicated in participating in the nucleosome remodeling process in Th1 and Th2 cells, respectively [256]. Also, transcriptional activity during different stages of Th1/Th2 polarization correlates with changes in histone acetylation of the *IL-4* and *IFN-γ* genes [256, 257].

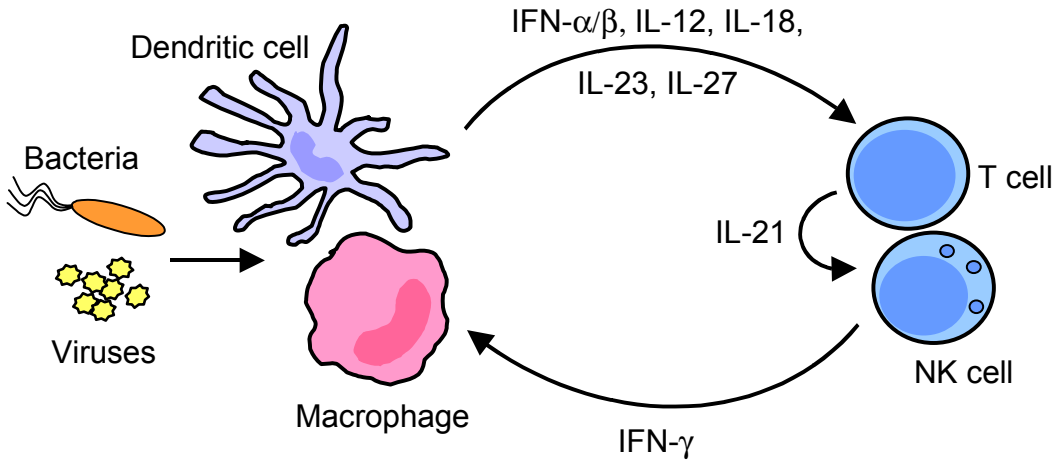

Figure 3. ***Cooperation of immune cells in microbial infections.*** During infection, DCs and macrophages recognize microbial structures and respond to them by producing a range of cytokines. In combination with cell–cell contacts, these cytokines activate NK and T cell effector functions. Activated NK and T cells also produce several cytokines, including IFN-γ. At the site of inflammation, IFN-γ enhances macrophage activation and antigen presentation to T cells.

3 AIMS OF THE STUDY

Cytokines guide the differentiation and functions of immune cells by regulating the activation and expression of specific transcription factors. Two transcription factor families, STATs and IRFs, are central in mediating cytokine responses. Genetic studies in mice have provided a wealth of information on how these transcription factors function in, and are essential for, cytokine responses. However, it is recognized that the function of the mouse immune system differs from its human counterpart in both innate and adaptive responses. Therefore, differences may exist also in the detailed mechanisms by which the cytokine signal in the cell is transmitted.

The aim of this study was to analyze cytokine-induced STAT and IRF transcription factor activity and gene expression in primary human blood cells involved in innate and adaptive immune responses.

The specific aims of the study were:

1. To characterize STAT and IRF expression and activation during differentiation of monocytes to macrophages or DCs.
2. To analyze the effect of cytokine stimulation on STAT and IRF expression and DNA-binding in mononuclear cells.
3. To elucidate the factors regulating the expression of IRF4 in mononuclear cells.

4 MATERIALS AND METHODS

4.1 Cell culture

Leukocyte-enriched buffy coats were obtained from voluntary healthy blood donors (provided by Finnish Red Cross Blood Transfusion Service, Helsinki, Finland). In each experiment, cells from 2-6 blood donors were used to minimize interindividual variation. Cells from different donors were cultured separately, and pooled when collected. In all cell cultures, RPMI 1640 medium was supplemented with penicillin (0.6 mg/ml), streptomycin (60 mg/ml), glutamine (2 mM), and HEPES (20 mM). Inactivated fetal calf serum (FCS; Integro, Zaandam, the Netherlands) was added to overnight PBMC cultures (5% FCS), and NK, T cell, and DC cultures (10% FCS). The purity of isolated cell populations was monitored by flow cytometric analyses with cell-type specific antibodies specified in references (I-IV). Doses and suppliers of cytokines and other reagents used to stimulate the cells are described in close detail in references (I-IV).

4.1.1 Isolation of PBMC (I- IV)

Human PBMC were isolated by density gradient centrifugation over a Ficoll-Paque gradient (Amersham Biosciences, Uppsala, Sweden). The cells were washed three times and cultured overnight in RPMI 1640 medium on six-well plates (Nunclon; $20x10^6$ cells/well).

4.1.2 Monocyte isolation and differentiation of macrophages (I, II, IV)

To isolate monocytes, purified PBMC were adhered onto plastic six-well plates (Falcon) for 1 hour at 37°C in RPMI 1640 medium ($10\text{-}20x10^6$ cells/well). After incubation non-adherent cells were removed and the wells were washed three times with cold PBS, pH 7.4. The cells were grown in Macrophage-SFM medium (Life Technologies) supplemented with antibiotics, and stimulated and harvested the next day. To obtain macrophages, monocytes were differentiated in Macrophage-SFM medium supplemented with antibiotics and GM-CSF (10 ng/ml) for 3-7 days. Fresh medium was replaced every two days. The purity of the macrophage population was > 90%.

4.1.3 Purification and differentiation of DCs (IV)

PBMC isolated by Ficoll-Paque gradient centrifugation were additionally centrifuged over a Percoll gradient and washed twice with RPMI 1640 medium. The cells were further purified by removing T and B lymphocytes using immunomagnetic beads (Dynabeads; Dynal, Oslo, Norway). After washing, cells were seeded on six-well plates (Falcon) and monocytes were allowed to adhere for 1 hour at 37°C in RPMI 1640 medium ($2.5x10^6$ cells/well). After washing once with PBS, cells were grown in RPMI 1640 medium with 10% FCS, GM-CSF (10 ng/ml), and IL-4 (20 ng/ml) for 3-7 days. Fresh medium (1 ml) was added every two days. At 7 days the purity the DC population was >90%.

4.1.4 Isolation and activation of T cells and polarization of CD4+ T cells (III)

Monocytes were removed from PBMCs by adherence and nonadherent T cells were further purified by nylon wool columns. Purified T cells were activated with 0.5 mg/ml immobilized anti-CD3 mAb (R&D Systems, Abingdon, UK) and cultured in RPMI 1640 medium supplemented with 10% FCS and 100 IU/ml IL-2 for 5-6 days. Cells were further expanded for 5-6 days with RPMI 1640 containing IL-2, at which point >99% of the cells were CD3 positive. In all experiments, the cells were removed from IL-2-containing medium before cytokine stimulations.

For lymphocyte polarization studies, lymphocytes were isolated from cord blood of healthy neonates and were further purified with magnetic beads (Dynal). Cells ($2x10^6$/ml) were cultured in Yssel's medium (Irvine Scientific, Germany) containing 1% AB-serum (Gemini Bioproducts, CA). Cells were primed with phytohemagglutinin (100 ng/ml; Murex Diagnostics, France), and grown in the presence of feeder cells (irradiated, CD32-B7-transfected mouse L-fibroblasts). For Th1 cultures, cells were supplemented with IL-12 (2.5 ng/ml), and for Th2 cultures with anti-IL-12 Ab (10 mg/ml) and IL-4 (10 ng/ml). After 48 h priming, IL-2 (40 IU/ml) was added into the cultures to enhance proliferation. The cells were cultured for 7 days.

4.1.5 Primary NK cells and NK-92 cell line (III)

NK cells were purified from nonadherent PBMCs by nylon wool columns and two-step density gradient centrifugation by Percoll (Amersham Biosciences), followed by depletion of monocytes and T and B lymphocytes with magnetic beads (Dynal).

NK cells were cultured in RPMI 1640 medium with 10% FCS. The purity of the NK cell population was > 90%.

Human NK-92 cell line [260] was maintained in continuous culture in MEM Alpha Medium (Life Technologies) supplemented with 12% horse serum (Life Technologies), 12% FCS (Integro), 0.2 mM i-inositol, 20 mM folic acid, 40 mM 2-mercaptoethanol, 2 mM L-glutamine, 0.6 mg/ml penicillin, 60 mg/ml streptomycin, and 100 IU/ml human rIL-2.

4.2 mRNA expression analyses

To study the influence of cellular differentiation or cytokine treatment on the cell population, changes in cellular gene expression were studied. Total cellular RNA was isolated either by the guanidium isothiocyanate/cesium chloride method [261, 262] or by using an RNA purification kit (Qiagen, Germany).

4.2.1 Northern blotting (I-IV)

Equal amounts of RNA were size-fractionated on 1% formaldehyde–agarose gels, transferred to a nylon membrane (Hybond, Amersham Biosciences), and hybridized with cDNA probes. Ethidium bromide staining of ribosomal RNA bands or hybridization with probes of housekeeping genes GAPDH or β-actin was used to ensure equal RNA loading. The probes were labeled with α-^{32}P-dATP (3000 Ci/mmol, Amersham Biosciences) by random priming. The membranes were hybridized at 42° (in buffer containing 50% formamide, 5x Denhardt's solution, 5x SSPE and 0.5% SDS; or in Ultrahyb; Ambion, Austin, TX), washed twice at room temperature and once at 60^{o}C in 1x SSC/0.1% SDS for 0.5 h each time, and exposed to Kodak AR X-Omat films at -70^{o}C using intensifying screens.

4.2.2 Quantitative RT-PCR (III)

cDNAs were prepared using Superscript II kit (Gibco). Primers and probes were designed using Primer Express software (Applied Biosystems, CA). Gene expression levels were measured using TaqMan RT-PCR (ABI Prism 7700, Applied Biosystems). Housekeeping gene EF1a was used for normalization of the results. The statistical significances of the differences between Th1 and Th2 samples were determined with the *t*-test.

4.2.3 cDNA microarray analysis (IV)

The human Immunochip cDNA microarrays were manufactured in the Finnish DNA Microarray Centre at Turku Centre for Biotechnology (www.btk.utu.fi). The ImmunoChip contains ~2000 genes implicated in immune cell activation and differentiation, including cytokines, chemokines and their receptors, transcription factors, and genes involved in signalling, apoptosis, and cell cycle regulation. All samples were labeled with FluoroLink™ Cy3-dUTP and Cy5-dUTP (Amersham Biosciences) using 16 μg total RNA for direct labeling during cDNA synthesis and hybridized using loop-design [263]. Separate images for Cy3 and Cy5 dyes were acquired, and gene transcript levels were determined from the fluorescence intensities of the scanned data image.

4.3 Protein expression analyses

In this study, two methods were employed to study the effect of differentiation and cytokine stimulation on protein expression and function. Direct Western blots were used to detect changes in protein expression levels. A more sensitive approach, immunoprecipitation, was used for studying protein–protein interactions and protein phosphorylation.

4.3.1 Western blotting (I-IV)

Aliquots of whole cell or nuclear extracts (10-30 mg protein) were separated on SDS-PAGE using the Laemmli buffer system. Proteins were transferred onto Immobilon-P membranes (Millipore, Bedford, MA). Binding of primary and secondary Abs was performed in PBS (pH 7.4) containing 5% non-fat milk for 1 h at room temperature. Antibodies used in immunoblotting are specified in the Materials and methods of original publications (I-IV). The protein bands were visualised on Hyper-Max film using the enhanced chemiluminescence system (Amersham Biosciences).

4.3.2 Immunoprecipitation (II)

Whole cell lysates were cleared by centrifugation and immunoprecipitated with specific antibodies using ProteinA–sepharose beads. After washing, the bound proteins were released in SDS sample buffer and equal aliquots were subjected to SDS-PAGE and Western blotting.

4.4 DNA –protein interaction studies

DNA–protein interactions on the chromatin level were studied by ChIP. In this method cells are fixed and their chromatin is sheared. Specific antibodies are used to precipitate pieces of chromatin containing particular proteins, and the occurrence of definite DNA fragments among the precipitates is detected by e.g. PCR. To study the specific interactions between transcription factor complexes and DNA elements found on gene promoters, two methods were employed: EMSA and DNA affinity binding assay. In these methods, both strands of the chosen DNA elements (Table 7) were synthesized with appropriate spacers (DNA Technology, Aarhus, Denmark). The oligonucleotides were annealed in 0.5M NaCl. For EMSA, nuclear extracts were prepared [264], and whole cell extracts were used for DNA affinity binding assays [265].

4.4.1 Chromatin immunoprecipitation (IV)

The level of histone acetylation in a promoter region correlates with the availability of the chromatin to the transcriptional machinery. Higher acetylation levels are associated with higher transcription rates. Detection of histone-H3 acetylation of the IRF4 promoter was done by using a commercial ChIP kit (Upstate Biotechnology, Lake Placid, NY) according to the manufacturer's instructions. Macrophages and DCs were treated as specified in the kit's protocol. The primers from the IRF4 gene promoter region used in PCR were 5'-ACAGCGCCTGGCCTATTTTG-3' (forward) and 5'-TGCATCTATTAGGCTGGTGA-3' (reverse). Input controls, representing the starting material prior to immunoprecipitation, were included in the PCR, alongside with the immunoprecipitated anti-acetyl-H3 samples and no-antibody control samples. PCR samples were analyzed by agarose gel electrophoresis on 1.5% gels.

4.4.2 EMSA (I-III)

The oligonucleotide was radioactively labeled by Klenow fill-in or by T4 polynucleotide kinase end-labeling. Nuclear protein/DNA binding reactions (RT, 0.5h) contained 2-10 μg nuclear extract protein. The samples were analyzed by electrophoresis on 6% non-denaturing low-ionic strength polyacrylamide gels in 0.25x TBE. The gels were dried and DNA/protein complexes were visualized by autoradiography. For identification of proteins contained in the complexes, samples were incubated with transcription factor-specific antibodies for 1 h on ice (supershift analysis).

4.4.3 DNA affinity binding assay (II-IV)

In this method biotinylated oligonucleotides were used and incubated with streptavidin–agarose beads (Neutravidin; Pierce, Rockford, IL) at +4°C for 2 h, in a ratio to yield maximum saturation of the beads with the oligonucleotide. Protein samples were incubated with these beads for 2 h at +4°C. After washing, the bound proteins were released in SDS sample buffer and equal aliquots were subjected to SDS-PAGE and Western blotting.

Table 7. ***Sequences of DNA elements used in EMSA and DNA affinity binding assays***

DNA element	**Sequence (5'→3')**	**Used in**
ISRE15	AGCTTGATCGGGAAAGGGAAACCGAAACTGAAGCCA	I
IRF1 GAS	AGCTTCAGCCTGATTTCCCCGAAATGACGGA	I, II, III
Pim-1 GAS	ACACACATCCCTTCCCAGAAATCAGGATTC	II
CIS GAS1	CCCCGTTTTCCTGGAAAGTTTTGGAAATCTGT	II
CIS GAS2	CCGCGGTTCTAGGAAGACGCTGCTTCCGGGAAGGGCTGG	II
IRF4 GAS	TTCCTATTTTCTTTTTAGTGAGTGCGATGTTCTCTAAACACCGC	III
λB	TCTTGGTTTCACTTCCTTTTATTTCTG	III
IRF4combi	GGCCATTTCCTATTTTCTTTTTAGTGAGTGCGATGTTCTCTAAACACCGC	IV
CD68	CCTCTCTTGGAAAGGAGGAAATGAAAGTC	IV
NF-κBRE1	AAAGTATGTAAAATCCCTGGTCCA	IV
NF-κBRE2	TCGGCTTGCAAAGTCCCTCTCCCC	IV
Stat6/κB	CCTCGCCCTTCGCGGGAAACGGCCCCAGTGACAGTCCCCGAAGC	IV

5 RESULTS AND DISCUSSION

In this study, leukocytes isolated from human peripheral blood were used as the cell model to study the effect of cytokines on the expression and functions of STAT and IRF transcription factors. Primary leukocytes are natural targets for cytokine action. In addition, several STATs and IRFs show cell type-specific expression patterns, with prominent expression particularly in hematopoietic cells. Thus, leukocytes provided the framework in which to study the biological functions of STATs and IRFs in their proper cellular context.

5.1 STAT and IRF expression in mononuclear cells

5.1.1 Up-regulation of STAT and IRF gene expression in response to IFNs (I)

In the beginning of this work, studies on STATs had concentrated on elucidating the molecular mechanisms and the identity of actual amino acid residues involved in JAK–STAT signaling. Only few reports had addressed the question of cytokine regulation of STAT and IRF gene expression [266, 267]. Expression and activation of STAT factors was shown to depend on the state of development and cellular differentiation [268]. This study was undertaken to clarify how IFN-stimulation affected the expression of IFN signaling molecules in human PBMCs and macrophages (I).

In most cells Stat1 and Stat2 are ubiquitously and constitutively expressed. In (I) we studied the expression of Stat1 and Stat2 using human PBMCs and monocyte-derived macrophages. IFN-α and IFN-γ were both able to strongly up-regulate Stat1 and Stat2 mRNA and protein expression (I, Figs. 1-6). In addition to STATs, also members of the IRF family take part in mediating IFN signal transduction. IRF9 (formerly p48) is a component of ISGF3, the IFN-α-specific signal transduction complex together with Stat1 and Stat2. IRF1 and IRF2 were at the time considered to be essential mediators of IFN responses that directly regulate type I IFN gene expression. IRF1 and IRF2 were considered to be an activator and a repressor of ISGF3/ISRE-mediated transcription, respectively. Expression of IRF1, and to a lesser extent IRF2 and IRF9, was up-regulated by IFNs in PBMCs and macrophages (I, Figs. 1-7). Increased expression of STATs and IRFs could not be blocked by inhibition of protein synthesis with cycloheximide (I, Fig. 8), suggesting that the effect of IFNs on STAT and IRF gene expression was direct.

Regulation of STAT gene expression in normal, non-transformed cells still has not been thoroughly investigated. Expression of IFN signal transducing components has been studied in various cancer cell lines, where retinoids and IFNs have been found to significantly increase expression of Stat1, Stat2, IRF1, or IRF9 [269-273]. The IRF9 promoter is reportedly regulated by c-myc, and by CCAAT/enhancer binding protein β in response to IFN-γ-treatment [274-276]. However, in these studies, induction of IRF9 expression was demonstrated to require *de novo* protein synthesis, contrary to the results in (I) where inhibition of protein synthesis did not block IRF9 mRNA expression. In most cell lines, basal IRF9 expression is very low or non-existing, contrary to the constitutive expression that is seen in primary mononuclear cells (I, Figs. 1, 3, 5, and 8). Expression of IRF9 protein itself may be required for direct IFN-induction of IRF9 gene expression.

The biologic effects of IRF1 are a result of inducible mRNA and protein expression [127], and the protein has a short half-life [277]. The IRF1 promoter contains a GAS-element [278, 279], and, accordingly, IRF1 gene expression was prominent in IFN-stimulated cells (I, Fig. 7), a finding also reported by several other studies [104, 105, 267]. In macrophages, IRF1 mRNA levels remained high for at least 24 h in response to IFN-γ-stimulation in contrast to IFN-α-stimulation where IRF1 mRNA levels peaked at 4 hours and declined thereafter. This may be indicative of prolonged Stat1 DNA-binding activity in response to IFN-γ-induction compared to IFN-α-induction. This phenomenon has been previously described in other cell systems as well [272, 280]. In this setting, prolonged expression of the IRF1 protein in response to IFN-γ could enhance IFN-induced gene expression by cooperating with the IFN-α-induced ISGF3 complex. Alternatively, IRF1 could function independently in regulating ISRE-mediated transcriptional activation after the ISGF3 complex has dissociated due to inactivation of its components by tyrosine dephosphorylation.

5.1.2 Stat5 expression during macrophage differentiation (II)

A central cell model in this study is the monocyte/macrophage differentiation model. GM-CSF is required for the generation of common monocyte-granulocyte precursors in the bone marrow, and M-CSF guides the growth of cells committed to the monocytic lineage. Both cytokines are used for *in vitro* generation of macrophages. In this study, GM-CSF, which activates the Jak2–Stat5 pathway, was used. Therefore, characterization of this signaling pathway during monocyte/macrophage differentiation was the intent of study (II).

The expression of Stat5A and Stat5B mRNAs was not significantly altered during differentiation of monocytes to macrophages (II, Fig. 8A). On the protein level,

however, two isoforms of both Stat5A (94 kDa and ~77kDa) and Stat5B (92 kDa and ~80 kDa) were detected in monocytes (II, Fig. 8B and 9A). In differentiated macrophages, only the 94- and 92-kDa forms were expressed (II, Fig. 8B and 9A). The expression of smaller forms of Stat5 proteins has been reported in several cell types, mainly in cells representing hematopoietic precursor populations [63, 91]. A nuclear protease has been identified that proteolytically processed Stat5A and Stat5B in the C-terminus [93]. Also, alternatively spliced mRNAs that could account for the expression of smaller protein forms have been reported for Stat5 [96, 281, 282]. These splice variants may be expressed in a cell type-specific manner, because in this study only single species of both Stat5A and Stat5B mRNAs were detected during monocyte-to-macrophage differentiation in Northern blot analyses. This suggests that if alternatively spliced mRNAs are expressed in these cells, their expression levels are very low. It may be that two different mechanisms, alternative splicing or proteolytic processing, may be used in parallel to generate smaller Stat5 forms in different cell types or in response to different stimuli.

Stat5A and Stat5B proteins were found to form both homo- and heterodimers in response to GM-CSF-stimulation (II, Fig. 9B). Heterodimer formation has been reported previously for Stat5A and Stat5B ([283]; reviewed in [284]). It may be that all types of dimers have their own target genes, as they have been shown to display subtle preferences in binding to different GAS element sequences [285, 286].

5.1.3 Expression of IRFs in NK and T cells (III)

Cell type-restricted expression pattern is characteristic to two IRF family transcription factors, IRF4 and IRF8. They are both expressed in the monocyte/macrophage lineage and in lymphocytes. Also, ubiquitously expressed IRFs, like IRF1, regulate the expression of many genes in a cell type-specific manner. IRF1, IRF4, and IRF8 have a role in initiating adaptive immune responses. Both IRF1 and IRF8 are essential for the production of IL-12 [131-133, 287, 288], a macrophage-derived cytokine that promotes the generation of Th1 immune responses. IRF1 is also needed for proper responsiveness of CD4+ T cells to IL-12 [288]. In addition to controlling CD4+ T cell functions, IRF1 regulates the differentiation of CD8+ T cells by affecting the expression of MHC class I molecules [289]. NK cell development also requires IRF1 because it controls the expression of the essential NK cell growth factor IL-15 by bone marrow stromal cells [290].

In humans, both IFN-α and IL-12 are able to promote IFN-γ expression by T cells and the generation of Th1 immune responses. These two cytokines are, together with IL-2 and IL-15, important activators of NK cells, and are able to induce the

production of IFN-γ by NK cells. In (III) the effect of IFN-α- and IL-12-stimulation on the expression of IRFs in NK and T cells was studied. IFN-α and IL-12 were able to stimulate IRF1, IRF4, and IRF8 mRNA and protein expression in NK-92 cells and primary NK cells (III, Fig. 1). In T cells, both IFN-α and IL-12 strongly up-regulated the mRNA expression of IRF1 and IRF4, but only IFN-α was able to induce significant expression of IRF8 mRNA (III, Fig. 2).

IRF1 is a well-known target gene for IL-12 in NK and T cells [291, 292], as shown also by our results. In (III) we showed that IFN-α and IL-12 caused enhanced mRNA expression and nuclear protein expression of IRF8 in NK cells. In mouse macrophages, it has been reported that IFN-α does not up-regulate IRF8 mRNA expression [293]. This is contradictory to our results in NK and T cells, and suggests that IRF8 expression in response to IFN-α is under cell type-specific regulation. In NK and T cells IRF4 mRNA expression and nuclear accumulation was enhanced by both IFN-α and IL-12 (III, Figs. 1 and 2). In B cells, expression of IRF4 is constitutive. Antigen receptor cross-linking [110] and PMA-stimulation or HTLV Tax-protein expression [111] induce IRF4 expression in mouse and human T cells, respectively. Our report (III) was the first to characterize the IRF4 gene as a cytokine-responsive gene in NK and T cells. Previously it has been shown that IRF4 mRNA levels were up-regulated in T cells of chronic myeloid leukemia patients and correlated with a favorable response to IFN-α therapy [294]. IFN-α and IL-12 are important cytokines in innate immune responses, as they activate IFN-γ production by NK cells. That these cytokines also induce IRF4 expression and DNA-binding in NK cells could indicate the involvement of IRF4 in regulating innate immune responses in addition to its role in adaptive T and B cell responses. To provide insight into this matter, NK cell functions should be evaluated in IRF4 knock-out mice.

IRF1 is important for the generation of Th1 type immune responses, and IRF4 is essential for T cell cytotoxic and antitumor responses, as well acquisition of Th1 and Th2 phenotypes [128, 129, 287, 288]. The expression of IRF1, IRF4, and IRF8 mRNAs was followed during *in vitro* polarization of cord blood T cells. Th1 polarizing conditions strongly favored the expression of IRF1 and IRF8 mRNAs compared to Th2 conditions (III, Fig. 3). These results support the role of IRF1 in Th1 cells. It is known that IRF8-deficiency leads to defective Th1 differentiation, but this results from the failure of APCs to produce IL-12. Our results suggest the possibility that IRF8 may play a role in Th1-differentiation on the T cell side as well. This question could be answered by studying IRF8 knock-out mice with targeted deletion only in T cells. The expression of IRF4 mRNA was more prominent in Th2 polarizing conditions (III, Fig. 3). Preferential expression of IRF4 detected in Th2 cells is supportive of the established role for IRF4 in controlling Th2

development [129, 148] and GATA-3 expression [129], but in our cell system, IRF4 mRNA was expressed also in Th1 polarized cells, albeit on a lower level than in Th2 cells. Interestingly, in IRF4-deficient mice, the differentiation of both Th1 and Th2 cells was compromised [129]. In contrast to IRF8-deficient mice, this effect was intrinsic to T cells, as proper Th differentiation was not observed when naive T cells lacking IRF4 were transferred to wild-type animals.

5.1.4 STAT and IRF expression during differentiation of monocytes into macrophages and DCs (IV)

Several reports have characterized, by large-scale microarray analyses, the differences in gene expression profiles between monocytes, macrophages, and DCs, either in the basal state or various infection models [295-301]. We were interested in the early changes in gene expression when monocytes are induced to differentiate into macrophages or DCs. Monocytes can be differentiated *in vitro* into macrophages by GM-CSF, or into DCs by GM-CSF together with IL-4. We analyzed monocytes stimulated with these cytokines by cDNA microarray analysis. As a part of this microarray project, IRF4 was found to be one of the most strongly up-regulated genes in response to GM-CSF- and IL-4-stimulation, and especially in response to the combination of these cytokines. Other IRFs did not show marked changes in their expression levels after stimulation (IV, Table 2). The strong induction of IRF4 mRNA was confirmed by Northern blotting (IV, Fig. 1). Cytokine induction of IRF4 expression has been reported in B cells, where IL-4 was found to up-regulate IRF4 mRNA expression [146]. In myeloid cells, (IV) is the first study reporting myeloid-specific cytokine-inducibility of IRF4 expression.

The expression of IRF1, IRF4, and IRF8 was more closely followed during macrophage differentiation. IRF1 expression was down-regulated during differentiation, and was not inducible by either GM-CSF or IL-4 (IV, Fig. 2). GM-CSF induced IRF4 mRNA expression only in monocytes, but IL-4 was able to up-regulate IRF4 expression also in 3-day macrophages (IV, Fig. 2). IRF8 mRNA expression was up-regulated by IL-4 but not by GM-CSF-treatment (IV, Fig. 2). Interestingly, a previous report showed that monocytes being differentiated into macrophages with GM-CSF became DCs when given IL-4 during the first 3-5 days of differentiation [302]. Our results also suggest that monocytes differentiating into macrophages retain the ability to respond to IL-4, and that IL-4-induced IRF4 mRNA expression could be a marker of a transcription factor phenotype typical for DCs.

The basal expression of IRF1, IRF4, and IRF8 mRNAs was studied in monocytes, macrophages, and DCs (IV). In the basal state, monocytes were the only cell type

expressing IRF1. IRF8 was constitutively expressed in all the cell types (IV, Fig. 3A). IRF8 is required for IL-12 expression by macrophages, for monocyte/macrophage and DC differentiation, and for control of myeloid cell proliferation [130-137]. Therefore, it is no surprise that IRF8 mRNA is basally expressed in all these cell populations. In contrast, constitutive IRF4 mRNA and protein expression was almost exclusively found in DCs (IV, Figs. 3A and 5C). The expression of IRF4 has previously been detected in mouse macrophages and human plasmacytoid DCs [138, 303, 304]. The discrepancies in the observed expression patterns between previous studies and (IV) probably reflect differences between species and cell models studied.

Expression of STAT genes was not significantly regulated in response to GM-CSF- or IL-4-stimulation, when measured by the cDNA microarray analysis. However, as the analysis covered only the immediate early events in monocyte differentiation into macrophages or DCs, the basal expression of STAT mRNAs in monocytes, macrophages, and DCs was studied. Stat1, Stat5A, and Stat5B mRNAs were constitutively expressed during differentiation (IV, Fig. 5A, see also II, Fig. 8). Stat6 expression was more prominent in monocytes and DCs. Clearly detectable Stat4 mRNA expression, however, was seen only in DCs (IV, Fig. 5A). The expression of Stat4 by activated monocytes, macrophages, or mature DCs in Th1 inflammatory conditions has been detected in other studies [305, 306], and our report showed expression also in immature DCs. In T cells the production of IFN-γ is absolutely dependent on Stat4-activation by IL-12 [77, 78]. APCs readily produce IFN-γ in inflammatory conditions [307-309], and a similar requirement on Stat4 expression and activity may operate in IFN-γ-production by these cells as well. [307-309]

5.2 Cytokine-induced STAT and IRF DNA-binding activity

5.2.1 IFN-γ pretreatment enhances the formation of IFN-α-induced DNA-binding complexes (I)

The expression of both reported splice variants for Stat1 (resulting in protein products p91 and p84) was up-regulated with comparable intensity by IFNs (I, Figs. 1, 3, and 5). The p84 variant lacks the serine residue involved in serine-phosphorylation in the transactivation domain, and is unable to support IFN-γ-induced gene activation [87]. However, in the context of the ISGF3-complex, p84 and p91 are functionally equivalent, due to the strong transactivation domain provided by Stat2 [310]. Stat1 dimers (termed GAF) were efficiently formed in response to both IFN-α- and IFN-γ-stimulation (I, Fig. 9 and 10). In our study (I),

the EMSA was not able to separate different combinations of p91/Stat1α and p84/Stat1β in Stat1 complexes. Thus, the activation potential of the detected Stat1 dimers cannot be assessed by this *in vitro* assay, and it is not possible to determine their functionality, distinct from the activity of ISGF3, in IFN-α responses. This would require the generation of Stat1α and Stat1β transgenic mice.

Stat1 dimers mediate signaling in response to both IFN-α and IFN-γ, and Stat1–Stat2 dimer together with IRF9 form the ISGF3 complex participating in IFN-α signaling. Accordingly, Stat1-deficient mice are extremely susceptible to several viruses due to defective IFN-α- and IFN-γ-signaling, and Stat2- and IRF9-deficient mice have defects in IFN-α/β-signaling [73, 74]. In (I) we found that IFNs enhanced the expression of IFN signaling components Stat1, Stat2, and IRF9. We further showed that when macrophages were primed overnight with IFN-γ followed by stimulation with IFN-α, enhanced formation of IFN-α-specific DNA-binding complexes, ISGF3 and GAF, was detected (I, Figs. 9 and 10). This may lead to enhanced expression of IFN-α target genes in the beginning of the innate immune response, when IFN-γ form NK cells and auto- or paracrine IFN-α from macrophages are induced by the invading microbe.

When other IFN combinations were used for priming and stimulation of cells, the formation of signaling complexes was significantly diminished (by IFN-γ/IFN-γ and by IFN-α/IFN-γ) or totally abolished (by IFN-α/IFN-α). Ligand-induced down-modulation of receptor cell-surface expression has been reported for both IFN-α and IFN-γ [311, 312]. At the time of study (I), negative regulation of STAT signal transduction pathways was thought to depend mainly on tyrosine phosphatases dephosphorylating the activated cytokine receptors, JAKs, and STATs. The same year, however, several groups reported characterization of a novel family of genes, now termed SOCS, whose expression is induced by activated STATs.

To date, SOCS protein family consists of eight members, CIS and SOCS 1-7. The expression of SOCS mRNA and proteins is rapidly induced by cytokine- or growth factor-stimulation of cells, and also by LPS and chemokines [20]. They contain SH2-domains, and are suggested to attenuate cytokine signaling by directly binding to phosphorylated tyrosines on cytokine receptor chains and JAKs, thereby preventing JAK activity or docking of STATs onto the receptor [17, 20]. Additionally, SOCS proteins function as ubiquitin E3 ligases, and target interacting proteins for proteasomal degradation. However, many of these studies are based on overexpression studies [19], and SOCS expression during normal cytokine stimulation has not been comprehensively characterized. In (I) we used various combinations of IFN-α and IFN-γ for priming and stimulation of macrophages, and studied the effect of these combinations on IFN signaling. In this experimental setting, the induction of SOCS gene expression by IFN-priming is likely to play a

role in down-modulating the subsequent signaling response to IFN-stimulation (combinations IFN-γ/IFN-γ, IFN-α/IFN-γ, and IFN-α/IFN-α). Indeed, the mRNA expression of SOCS3 and CIS is induced in macrophages following stimulation by IFN-α or IFN-γ, providing at least a partial explanation for the difference in signaling efficiency in response to various IFN combinations detected in human primary macrophages (Fig. 4).

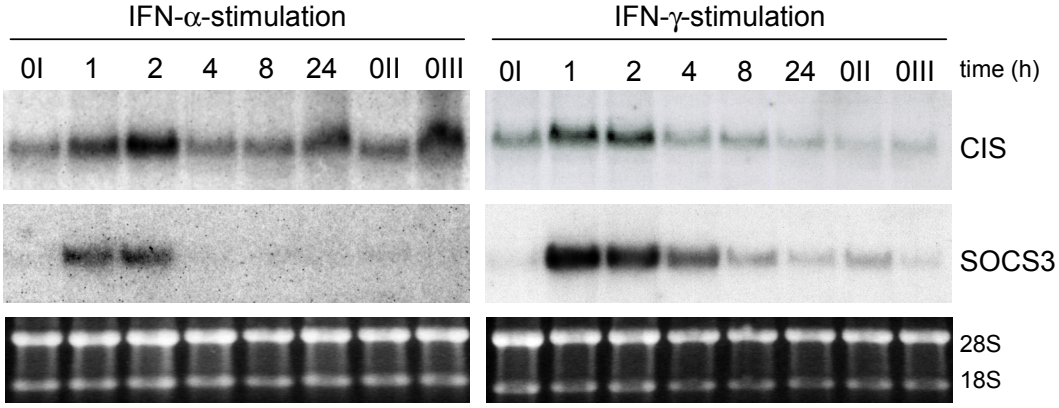

Figure 4. ***SOCS3 and CIS mRNA expression is induced by IFN-α and IFN-γ in human macrophages.*** Macrophages were stimulated with IFN-α or IFN-γ for times indicated in the figure or left untreated, and total cellular RNA was isolated. 10 μg samples of total RNA were run on gels and analyzed by Northern blotting with CIS and SOCS3 probes. 0I, 0II, and 0III represent untreated samples collected at 1, 4, and 24 hours. Ethidium bromide staining of ribosomal RNAs is shown as a control of equal loading.

5.2.2 GM-CSF-induced JAK–STAT signaling in differentiating macrophages (II)

Both GM-CSF and M-CSF can be used to generate macrophages *in vitro*. These two growth factors use different receptor systems. GM-CSF uses a type I cytokine receptor, with a ligand-specific α-chain and the common β_c-signaling chain. M-CSF utilizes a receptor with intrinsic tyrosine kinase activity. When studying GM-CSF-induced Jak2–Stat5 activation (II), we discovered that GM-CSF-induced Stat5 DNA-binding activity was considerably reduced in macrophages compared to monocytes (II, Figs. 1 and 5). This was also reflected on the level of mRNA expression of two known Stat5 target genes in macrophages (II, Fig. 3). Continuous administration of GM-CSF might desensitize the cells and decrease GM-CSF-induced Stat5-activation in macrophages. To investigate this phenomenon, M-CSF

instead of GM-CSF was used for macrophage differentiation, and cells were then stimulated with GM-CSF. Identical results were obtained from M-CSF-differentiated cells: GM-CSF-induced Stat5 DNA-binding and target gene mRNA expression were both decreased in macrophages (II, Fig. 4). In addition, mRNA or protein expression of GM-CSF receptor components or Jak2 was not significantly affected by macrophage differentiation (II, Fig. 7). Jak2 activity, measured by GM-CSF-induced tyrosine phosphorylation, was actually higher in macrophages that in monocytes (II, Fig. 7). The results in (II) strongly suggested that the decreased responsiveness of macrophages to GM-CSF is regulated on the level of Stat5, and is not caused by down-regulation of the GM-CSF receptor system in these cells.

Another interesting feature of GM-CSF-induced Stat5 DNA-binding was the finding that the DNA-binding complex in monocytes had faster mobility in EMSA gels than the respective macrophage complex (II, Figs. 1, 4-6). Supershift EMSA studies with Stat5-specific antibodies confirmed that the DNA-binding complexes consisted of only Stat5 (II, Fig. 5D). By DNA affinity binding assays we were able to show that the 77/80 kDa Stat5 isoforms also participated in the formation of the DNA-binding complex (II, Fig. 9C). The expression of smaller Stat5 isoforms has been linked to repression of target gene expression in hematopoietic precursor cells expressing exclusively the truncated forms with no full-length Stat5 protein expression [63, 91]. The results in (II) do not fully support the concept of target gene repression by smaller Stat5 isoforms, as the participation of the 77/80-kDa forms in the DNA-binding complexes did not inhibit *CIS* and *pim-1* expression in monocytes. This may be due to the co-expression of the full-length forms, but recent evidence also suggests an independent role for the β-forms of STATs in gene regulation [89, 90] as opposed to a sole function as dominant negative variants of the α-forms.

In (II) we used the mRNA expression of two known Stat5-responsive genes, *pim-1* and *CIS* [313-315], as markers for GM-CSF activity in stimulated cells. Pim-1 is a serine/threonine kinase involved in controlling myeloid cell proliferation [313, 316], and may be associated with proliferative responses in monocytes. Our results show that its expression was strongly reduced in macrophages compared to monocytes (II, Fig. 3 and 4B). In addition to the Jak2–Stat5 pathway, GM-CSF activates the ERK MAPK-pathway and phosphatidylinositol-3-kinase pathway [317]. One main function of these signaling pathways is to transmit survival signals in hematopoietic cells. By reducing activation of Stat5 in response to GM-CSF stimulation cells may control the expression of some proliferation-associated genes, like pim-1, when cells are terminally differentiated. At the same time, signaling components upstream of Stat5 are left intact, allowing for the transmission of GM-CSF survival signals into the cell.

CIS belongs to the SOCS family of inhibitors, and it blocks the binding of Stat5 to the cytokine receptor and hence the activation of Stat5 [315, 318]. However, in monocytes the expression of CIS mRNA was strongly up-regulated already at 1 h after GM-CSF-stimulation, and remained on the same high level for at least 24 h, displaying no clear signs of inhibition of Stat5 DNA-binding activity (II, Fig 3). CIS is a ubiquitinated protein [319], and may target activated receptor chains to proteasomal degradation. In differentiating monocyte/macrophages this down-regulation is apparently transient, as both cell types readily express GM-CSF receptor α- and β-chains (II, Fig. 7). The reduced activation of Stat5 caused by CIS expression might be compensated by a slower off-rate of Stat5β-containing dimers from DNA, and might lead to prolonged Stat5 target gene expression in monocytes. The smaller β-isoforms of Stat3 and Stat5 have been reported to display enhanced DNA-binding activity [98, 320], thus giving support to this hypothesis.

Interestingly, in EMSA gels a complex with very low mobility was detected in monocytes stimulated with GM-CSF for 0.5h (II, Figs. 1, 4A, 5, 6). This complex most likely corresponds to formation of Stat5 tetramers. STAT tetramers reportedly form through N-terminal protein–protein interactions [69, 70] on promoters containing several GAS elements. Tetramerization of Stat5 has been reported to occur on the *CIS* promoter used in this study [286, 315] and on the *IL-2* promoter [321]. Tetramerization is especially important on tandem GAS sites possessing low STAT binding affinity [13].

5.2.3 IFN-α and IL-12 induce IRF4 and IRF8 DNA-binding in NK and T cells (III)

IRF proteins form complexes with other IRFs as well as other transcription factors. IRF1 is generally regarded as a transcriptional activator, but IRF4 and IRF8 have dual roles as activators and repressors of gene expression [127, 139, 322]. Their function depends on the interacting partners and the target DNA element. Both IRF4 and IRF8 interact with PU.1, an Ets-family transcription factor expressed in several hematopoietic cell lineages. In the context of Ets/IRF sites, IRF4 and IRF8 function as transcriptional activators. Known PU.1/IRF targets in lymphocytes include enhancers of Ig κ and λ chains and IL-1β, and the promoter of CD20 [323].

IFN-α and IL-12 up-regulated IRF4 and IRF8 expression in NK and T cells (III). This was also reflected by enhanced nuclear expression of IRF4 and IRF8 proteins. Next, the DNA-binding of these transcription factors was studied. In NK cells, IFN-α and IL-12 enhanced DNA-binding of IRF4 and IRF8 (III, Fig. 4A). In T cells similar results were obtained, but the response to IL-12 was weaker compared to that of IFN-α. Stimulation of the cells via TCR crosslinking was the strongest inducer of

IRF4 and IRF8 DNA-binding (III, Fig. 4B). Interestingly, even though IL-12 did not enhance IRF8 mRNA expression in T cells, IRF8 DNA-binding was weakly increased in T cells at 6 h after IL-12-stimulation, suggesting that IL-12 has an enhancing effect on IRF8 target gene regulation.

IRF4 and IRF8 are most likely able to bind DNA without specific activation by receptors, although tyrosine phosphorylation does regulate some aspects of IRF8 DNA-binding [152]. Therefore, coordinately up-regulated expression is one means to enhance the expression of IRF4/IRF8 target genes [129]. To date, IRF4/IRF8-specific target genes involved in Th responses have not been identified, but comparative microarray gene expression analyses between wild-type and IRF4- or IRF8-deficient mice should clarify the role of these transcription factors in different aspects of immune responses.

5.3 Control of IRF4 gene expression

IRF4 has, until recently, remained one of the least studied members of the IRF family. Initial studies reporting IRF4 expression described it as a lymphocyte-specific IRF. It is constitutively expressed in B cells, and in T cells its expression is activated by antigen receptor cross-linking and expression of the oncogenic HTLV Tax-protein. The analyses of IRF4-deficient mice revealed that IRF4 is required for the maturation of B cells and generation of plasma cells [128]. T cell proliferation was reduced, and T cell cytotoxic activity was absent in these mice [128]. Furthermore, Th1 and Th2 responses were dysregulated [129]. Later, the expression of IRF4 was also detected in the cells of the myeloid lineage, namely monocytes and macrophages [138, 303], coinciding with the expression pattern of IRF8. Only a few myeloid IRF4 target genes are presently known [322-324]. Perhaps due to incomplete expression data available at the time, the question of IRF4 functions in DCs was not address by the original knock-out study. Very recently, IRF8 was reported to take part in TLR9/NF-κB signaling in DCs [325]. Regarding the close relationship and similar expression patterns of IRF4 and IRF8, involvement of IRF4 in TLR signaling should be examined, as well as the possible DC defects in IRF4 deficient mice.

IRF4 promoter analyses in human and mouse describe binding sites for Sp1, AP-1, NF-κB, and PU.1 [110, 326, 327]. A later report identified an additional NF-κB-binding site and a CD28 responsive element in T cells [328]. DNA elements conferring cytokine-inducibility of the IRF4 promoter were not reported by these studies. In (III) we showed that IRF4 expression in NK and T cells was rapidly up-regulated by IFN-α and IL-12, suggesting that cytokine-inducible promoter elements exist in the IRF4 promoter. Also our own microarray analyses pointed out *IRF4* as a

gene specifically up-regulated by cytokines relevant to generation of macrophages and DCs (IV, Table 2, Figs 1-3A). This prompted us to analyze the constitutive and cytokine-inducible expression of the cell type-restricted IRFs in our cell model in more detail (III, IV).

5.3.1 The IRF4 promoter contains a GAS-like DNA element able to bind Stat4 in response to cytokine stimulation in NK and T cells (III)

In (III), while studying cytokine-induced expression of IRF4, we identified a GAS-like DNA element in the IRF4 promoter (III, Fig. 5). The IRF4 GAS showed specificity in its STAT-binding pattern. Stat4, but not Stat1 or Stat3, bound to this element in response to IFN-α and IL-12 stimulation, even though Stat1 was efficiently activated by IFN-α (III, Fig. 5). Binding of Stat4 to this site was strictly cytokine-inducible. Of the other IRF genes, at least the promoters of IRF1, IRF7, and IRF8 are known to contain a GAS element [267, 329, 330], and these genes are also IFN-inducible. Additionally, IRF9 mRNA expression is up-regulated by both IFN-α and IFN-γ (I), although the mechanism is likely to be different, as no GAS elements have been identified in the IRF9 promoter. Taking into consideration the fact that several IRFs function as dimers in the regulation of their target genes, coordinated expression of these proteins is probably of importance. This is emphasized by the findings reporting that the same cytokines or STAT proteins control the expression of many IRFs.

5.3.2 Detection of histone H3 acetylation levels in the IRF4 promoter in macrophages and DCs (IV)

IRF4 mRNA was constitutively expressed in DCs but no expression could be detected in macrophages, (IV, Fig. 3A), suggesting that IRF4 gene expression might be regulated at the level of differential promoter accessibility in macrophages and DCs. When histones on a promoter region are acetylated, transcription rate from this promoter is higher than from promoters associated with non-acetylated histones. We performed ChIP experiments using anti-acetyl-histone H3 antibodies to detect differences in IRF4 promoter acetylation between macrophages and DCs. The results showed that, from both macrophages and DCs, IRF4 promoter-specific primers produced a PCR product of the expected size after immunoprecipitation with anti-acetyl-histone H3 antibodies (IV, Fig. 3B). These results suggested that differences in IRF4 promoter histone acetylation do not explain the difference in IRF4 mRNA expression between macrophages and DCs. How, or if, cytokine

stimulation affects IRF4 mRNA expression in macrophages remains to be elucidated, but our results showed that at least IL-4-stimulation did not induce IRF4 mRNA expression in these cells (IV, Fig.2).

5.3.3 IRF4 promoter analysis

The MatInspector program [331] was used to localize putative transcription factor binding sites in the IRF4 promoter region (IV, Fig. 4) of both human and mouse origin. The analysis was done in parallel because we wanted to assess evolutionary conservation of putative transcription factor binding sites predicted by the program. Mouse and human promoters have been partially characterized previously [110, 326-328]. Sequence analysis yielded several transcription factor binding sites that had not been previously reported. Among those relevant to cytokine stimulation, we chose three putative NF-κB binding sites, one of which is located adjacent to a conserved Stat6-binding site. In addition, we analyzed the functionality of an extended IRF4 GAS element. The IRF4 GAS element was extended 8 nucleotides upstream (named IRF4combi) because of the predicted presence of a PU.1/IRF4 binding site adjacent to the GAS element. Of note, the MatInspector program did not recognize the functional IRF4 GAS element as a putative STAT binding site.

STAT binding to the Stat6/κB and IRF4combi elements

The GAS element, originally characterized as an IFN-γ-activated sequence that binds Stat1 dimers, is found in the promoters of cytokine-inducible genes, and is able to bind various STAT dimers. The GAS consensus sequence is $TTCN_{2-4}GAA$. However, some GAS elements show preferential binding of distinct STAT dimers while nearly completely excluding the binding of others. Stat6 dimers bind with high affinity to GAS sites where the half-sites are separated by four nucleotides (N_4-sites), but are also able to bind to the more common N_3-sites [13]. Our computer analysis (IV, Fig. 4) and study (III) suggested the presence of two putative GAS sites, and the binding pattern of different STAT dimers to these sites was studied in DCs (IV).

The cytokines used in *in vitro* differentiation of macrophages and DCs, GM-CSF and IL-4, activate Stat5 and Stat6, respectively. Stat5 and Stat6 DNA-binding was detected only in 7-day DCs (IV, Fig. 5B). The Stat6/κB element bound Stat6 very strongly, suggesting that Stat6 is, as predicted, the major factor binding to this site (IV, Fig. 5B). Stat6 and NF-κB are known to interact on similar DNA elements [332-334]. Stat6 is activated by IL-4 in a wide variety of human cell types, including DCs, B cells, and Th2 cells, where IRF4 mRNA was strongly expressed during early

phases of IL-4-driven differentiation (III, Fig. 3) [146]. Interestingly, Stat6 binding to the Stat6/κB site was strong in 7-day DCs, two days after the last input of GM-CSF/IL-4. Similar prolonged Stat6 DNA-binding activity has been reported in murine B cells [335] during continuous IL-4-stimulation. This experimental setting is analogous to the use of IL-4 in the culture medium during differentiation of DCs in our cell system and suggests that in DCs, IL-4 elicits a sustained Stat6 DNA-binding response, even though STAT DNA-binding activity is usually of short duration and actively terminated by phosphatases and regulatory proteins. Maintaining continuous Stat6 DNA-binding activity could provide a mechanism by which Stat6 regulates constitutive target gene expression. In B cells, IL-4-stimulation up-regulates IRF4 mRNA expression [146], but this report did not describe promoter elements that could bind IL-4-activated Stat6. The IRF4 promoter Stat6/κB element described in (IV) may support both constitutive and inducible IL-4 responsiveness in different cell types.

Basal Stat4 mRNA expression was considerably stronger in DCs than in macrophages (IV, Fig. 5A). Accordingly, basal Stat4 DNA-binding to both GAS elements was detected only in DCs (IV, Fig. 5B). However, phosphorylation of Stat4 could not be directly detected in DCs (data not shown). Nonphosphorylated Stat1 and Stat3 are constitutively found in the nucleus of unstimulated primary human cells and cell lines [336, 337]. Interestingly, constitutive transcription of *LMP2* gene is supported by nonphosphorylated Stat1 together with IRF1 [15], and constitutive expression of certain caspases depends on the presence of Stat1, not requiring its activation or dimerization [14]. Stat4 could reside in the nucleus in nonphosphorylated form as well, and participate in basal IRF4 gene regulation in an analogous manner. Nonphosphorylated STAT proteins may emerge as common mediators regulating constitutive gene expression.

NF-κB binding to IRF4 promoter NF-κB elements in DCs

Several NF-κB sites have been characterized in the human and mouse IRF4 promoters [110, 327, 328], and our computer analysis (IV, Fig. 4A) of the human IRF4 promoter region revealed the presence of additional putative NF-κB binding sites (IV, Fig. 4). NF-κB(p50) and NF-κB(p65) mRNA expression was lower in macrophages than in DCs, and the same difference applied to constitutive NF-κB DNA-binding (IV, Fig. 6). Similar results have been obtained by others [338, 339].

Basal binding of NF-κB(p50) protein to Stat6/κB element was detected in all cell types (IV, Fig. 6B). NF-κB(p50) homodimers are associated with transcriptional repression [340, 341]. More differentiated cells expressed a higher molecular weight form of NF-κB(p50) protein compared to monocytes (IV, Fig. 6B). This shift in

molecular weight could be due to post-translational modifications of NF-κB(p50). Phosphorylation and acetylation of NF-κB subunits have been reported to occur [227, 342, 343]. Phosphorylation of NF-κB subunits controls the DNA-binding and gene regulatory activity of NF-κB homo- and heterodimers. Basal binding of NF-κB(p65), RelB, and c-Rel was only detected in differentiated macrophages and DCs.

Inducible binding of NF-κB proteins to the IRF4 promoter NF-κB elements in TNF-α-stimulated DCs was also studied in (IV). Using three different oligonucleotides containing putative NF-κB binding sites (IV, Fig. 4A) we could show that NF-κB(p50), RelB, and c-Rel constitutively bound to all of these three elements, with some enhanced binding after TNF-α-treatment (IV, Fig. 7A). Strong NF-κB(p65) binding was seen in TNF-α-treated DCs (IV, Fig. 7A). DCs display high constitutive expression of IRF4 mRNAs (IV, Fig. 3A), and TNF-α stimulation only weakly increased the expression of IRF4 mRNA (IV, Fig. 7B). In lymphoid cells, constitutively active RelB mediates basal transcription [344, 345]. RelB is also essential for the generation of myeloid DCs in the mouse [346]. Constitutive expression and DNA-binding activity of RelB may be one of the factors regulating basal IRF4 expression in DCs in a cell type-specific manner.

IRF4 may autoregulate its own gene expression via the Ets/IRF element in the IRF4 promoter

The PU.1/IRF4 element predicted by the MatInspector analysis was found to be functional in DNA affinity binding studies (IV, Figs. 5 and 7). IRF4 bound to this element only in 7-day DCs (IV, Fig. 5C), although IRF4 protein expression was high also in 3-day DCs (IV, Fig. 5C). Basal DNA-binding of IRF4 to the IRF4combi and CD68 gene Ets/IRF DNA elements was detected in DCs (IV, Fig. 7C), and the binding was enhanced by TNF-α-treatment. IRF8 gene expression is also reported to be autoregulated by its own gene product [293]. The fact that both IRF4 and IRF8 proteins bind to their own promoter regions suggests that autoregulation of gene expression is one means of maintaining cell type-restricted expression of these IRFs.

PU.1, an Ets-family transcription factor, is known to form a gene regulatory complex with IRF4 [323]. PU.1 mRNA expression remained unchanged during differentiation of macrophages and DCs (IV, Fig. 3A). PU.1 bound to the DNA element in 7-day macrophages and DCs, and stronger binding was seen in DCs (IV, Fig. 5C). PU.1 is able to bind DNA on its own, but IRF4 binding to Ets/IRF sites requires PU.1 [109]. In DNA affinity binding studies, a smaller form of PU.1 was consistently detected in monocytes and 3-day cells (IV, Fig. 5C). PU.1 protein contains a PEST domain rich in proline, glutamic acid, serine, and threonine residues. PEST sequences are thought to be involved in regulating protein stability, and proteins carrying this sequence are targeted for rapid proteolytic degradation

(reviewed in [347]). However, in the case of PU.1, it is suggested that this region is involved in protein–protein interactions and does not function as a degradation signal [348]. In the case of interaction between PU.1 and IRF4, a specific serine residue in the PEST region of PU.1 needs to be phosphorylated [349]. In our cell system the smaller form of PU.1 was able to bind to DNA, but was apparently unable to interact with IRF4 despite IRF4 expression in 3-day DCs. It has previously been observed that mere DNA-binding domain interactions between PU.1 and IRF4 are not strong enough to support formation of the PU.1/IRF4 DNA-binding complex [348, 350, 351]. These results suggested that the smaller PU.1 detected in our cell system lacks the region necessary to support the PU.1–IRF4 interaction. ChIP analyses on PU.1 and IRF4 binding on the IRF4 promoter should clarify the roles of these factors in successive stages of macrophage and DC differentiation.

6 CONCLUDING REMARKS

The immune system is a network of cells, receptors, and soluble mediators. Immune cells are continuously renewing and responding to pathogenic challenges during the host's lifetime. Their gene expression profiles undergo dramatic changes throughout the development, differentiation, and cell cycle. Transcription factors are the proteins responsible for coordinating these complex processes, from cellular differentiation and cell type-specific phenotypes to active proliferation during immune responses. It has been estimated that 5% of the genes in the human genome encode transcription factors, which underscores the importance of transcriptional control in all cellular processes.

The focus of this work was to characterize STAT and IRF transcription factor gene expression and activation in human immune cells. STATs and IRFs regulate cytokine-induced signaling in immune cells, and they themselves are cytokine target genes. In this study, STATs and IRFs were studied in two different experimental settings: normal cellular differentiation and stimulation of cells with inflammatory cytokines IFN-α, IFN-γ, and IL-12.

When monocytes differentiate into macrophages, Stat5 activation and target gene expression in response to growth factor stimulation is significantly reduced. Different Stat5 isofoms were expressed in monocytes and macrophages. The interactions of STAT β-isoforms with other transcription factors and transcriptional coactivators in all probability differ from the full-length forms, providing an additional level of specificity to gene regulation by STATs. DCs and macrophages differ in their expression of IRF4 and Stat4. These transcription factors are likely to contribute to the generation of the DC phenotype. Also, determining the role of IRF4 in TLR signaling could provide important insights into the differential role of DCs and macrophages during innate immune responses.

Stimulation of macrophages and NK and T cells with inflammatory cytokines resulted in up-regulation of Stat1, Stat2, IRF1, IRF4, IRF8, and IRF9 gene expression. Cytokine induction of IRF gene expression could strengthen and prolong the effect of cytokines in immune cells, and also induce the expression of an expanded set of target genes. Cytokine cascades are important in immune responses. The expression of many cytokine target genes is turned on only when several signaling pathways, like STATs, IRFs, and NF-κB, are activated simultaneously. This is exemplified also by the IRF4 promoter, which harbors binding sites for all these factors. The immune system is a network of signals, and cytokine signaling is mediated by a network of transcription factors.

The ability to mount an effective immune response, which includes generation of immune cells of the required types, appropriate cytokine milieu, and proper temporal regulation, is essential for the survival of the organism. Activation of different transcriptional systems by microbes and cytokines and subsequent down-regulation of these responses are central factors in maintaining host homeostasis.

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