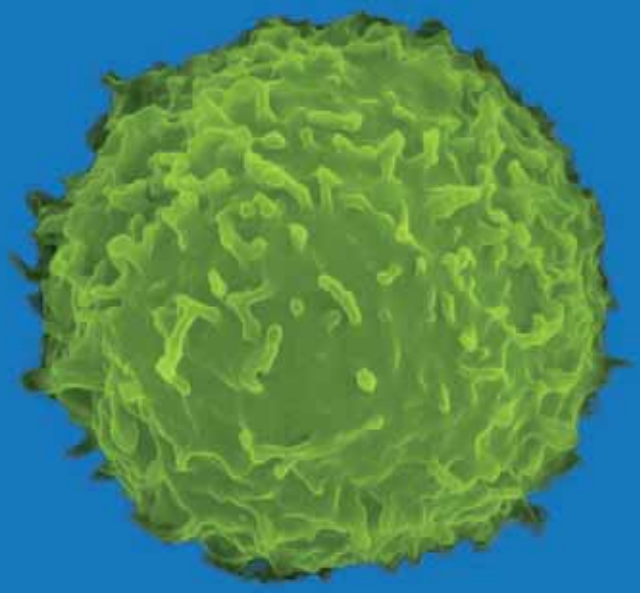

Mari Strengell

Role of IL-21 in Regulation of Leukocyte Functions

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Department of Viral Diseases and Immunology
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Helsinki, Finland
and
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Division of Genetics,
University of Helsinki, Finland
and
Helsinki Graduate School in Biotechnology and Molecular Biology

Mari Strengell

ROLE OF IL-21 IN REGULATION OF LEUKOCYTE FUNCTIONS

ACADEMIC DISSERTATION

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Department of Viral Diseases and Immunology, National Public Health Institute, Helsinki, Finland

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Helsinki Graduate School in Biotechnology and Molecular Biology

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ABSTRACT

Immune system protects body from invading pathogens and other microbes. The immune system consists of white blood cells, leukocytes, which form a complex network along with soluble mediators, cytokines, by which the cells communicate with each other. In addition to secreted soluble mediators, the cells have also direct interactions with each other via their cell surface receptors. The immune system can be divided into two parts, innate and adaptive immunity. Innate immunity, which consists of complement system, phagocytic cells, e.g. granulocytes and macrophages, and natural killer cells, are activated within the first hours after infection. The activation of innate immunity does not require specific antigen recognition but the recognition of conserved microbial structures or molecules (e.g. dsRNA) that are abundant in microbes but missing in the host. These structures are recognized by for example Toll-like receptors (TLR). On the contrary, adaptive immunity includes the specific antigen recognition by B and T cells. B cells can bind antigens directly but T cell activation requires presentation of the antigen by macrophages or dendritic cells in association with MHC molecules. After antigen recognition T (and B) cells get activated, proliferate and start to destroy infected cells.

Effective communication between the cells of the immune system is essential for the proper function of the whole complex network. Cells secrete cytokines that mediate signals between the cells. These signals lead to alterations in the gene expression of the target cell. Tens of different cytokines can be divided into several groups based on their structure, function, or the structure of the receptors they are binding. Common gamma-chain receptor family contains the cytokines IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. IL-21 is the newest member of this family. It is produced by activated T cells and it affects the function of T cells in many ways. IL-21 also enhances the cytotoxic activity of NK cells towards e.g. virus-infected cells. In this study the regulatory role of IL-21 on human primary T and NK cells was studied. The effect of IL-21 on the regulation of certain Th1 and innate immunity genes was analyzed. We found out that IL-21 upregulated the expression of IL-2R, IL-12R, and IL-18R in NK and T cells, which enhaces the responsiveness of these cells to Th1 cytokines. Further IL-21 enhanced the gene expression as well as protein production

of IFN-γ. IFN-γ production was synergistically enhanced by IL-21 and IL-15 co-stimulation. Also IL-21 and IL-18 synergistically enhanced NK and T cell IFN-γ production.

Activation of gene expression requires the activation and binding of transcription factors to the promoter of the target gene. Janus kinases (Jak) and Signal transducer and activator of transcription (STAT) form an important signaling pathway, which is activated by cytokine stimulus. IL-21 stimulation of NK and T cells preferentially activated STAT3. Also STAT3-associated Jak1 and Jak3 kinases were activated. IL-21 induced STAT1 and STAT4 activation and DNA binding too. This could be verified both by detecting the induced tyrosine phosphorylation of Jak and STAT proteins and analyzing the binding of STATs to the promoter of several target genes e.g. IL-2Rα.

IFN-α/β is produced during virus infection by macrophages and dendritic cells. IFN-α/β has direct anti-viral effects but it also enhances NK cell cytotoxic activity and T cell activation thus promoting both innate and adaptive immune responses. The regulatory role of IFN-α/β on IL-21 production from T cells was analyzed. IFN-α/β enhanced the expression of IL-21 in T cells. A sequence analysis of IL-21 promoter revealed a putative STAT binding element, which indeed bound STAT proteins after IFN-α stimulation. IFN-α/β also negatively regulates IL-21 functions by down-regulating IL-21R expression in NK and T cells. This reduced responsiveness to IL-21 leads to inhibited STAT3 activation by IL-21.

Dendritic cells present antigens to T cells and are thus an important part of adaptive immunity. Dendritic cells can be obtained *in vitro* from primary monocytes by stimulating cells with GM-CSF and IL-4. In differentiating DCs the expression of IL-21R was induced but it remained at very low level in differentiating macrophages. Differentiated DCs are immature until they meet microbe or their component, such as LPS. Mature DCs up-regulate the expression of HLA and CD86 surface molecules and secrete cytokines and chemokines. Pretreatment of DCs with IL-21 inhibited LPS-induced DC maturation and cytokine production. LPS is recognized by TLR4 but we could not observe any reduction in the expression of TLR4 or its signaling components after IL-21 stimulation. However, IL-21 induced the expression of SOCS1 and SOCS3 genes. SOCS1 has previously been reported to inhibit LPS-induced TLR4 signaling and DC maturation.

The results obtained in this study demonstrate that IL-21 has and important regulatory functions in human NK and T cells and DCs.

Keywords: Immune system, leukocyte, cytokine, gene expression

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TIIVISTELMÄ

Immunijärjestelmän tehtävänä on suojata elimistöä ympäristössä esiintyviltä taudinaiheuttajilta ja muilta mikrobeilta. Veren valkosolut eli leukosyytit sekä valkosolujen erittämät viesti- ja välittäjäaineet eli sytokiinit muodostavat pääosan immunijärjestelmästä. Tämän lisäksi immunijärjestelmään kuuluu myös komplementtisysteemi, joka on tärkeä luonnollisen immuniteetin osa. Immuniteetti voidaan jakaa kahteen osaan. Synnynnäinen immuniteetti, johon kuuluvat komplementin lisäksi patogeeneja fagosytoivat makrofaagit ja neutrofiilit sekä luonnolliset tappajasolut (NK), on ensimmäisenä aktivoituva immunijärjestelmän osa. Synnynnäisen immuniteetin aktivaatio ei vaadi spesifistä antigeenin tunnistusta vaan vieraan mikrobin pintarakenteet tai sen elinkierrossa syntyvät molekyylit, esim. kaksijuosteinen RNA, tunnistetaan vieraaksi mm. Toll-like reseptorien (TLR) avulla. Synnynnäisen immuniteetin aktivaatio tapahtuu nopeasti muutaman tunnin tai päivän kuluessa, jona aikana adaptiivinen immunijärjestelmä ehtii myös aktivoitua. Adaptiivisen eli antigeenispesifisen immuniteetin aktivoituminen edellyttää, että mikrobin fagosytoinut makrofaagi tai dendriittisolu esittelee prosessoimansa mikrobin peptidejä (antigeeneja) T- ja B-soluille. T- ja B-solut tunnistavat antigeenin, jakautuvat ja alkavat tuhota taudinaiheuttajia.

Immunijärjestelmän tehokkaan toiminnan kannalta on tärkeää, että solut voivat viestiä keskenään tehokkaasti ja tarkoituksen mukaisesti. Sytokiinit ovat solujen tuottamia proteiineja, jotka reseptoriinsa sitoutuessaan, saavat kohdesolussa aikaan muutoksia geeninsäätelyssä. Sytokiineja on kymmeniä ja ne voidaan rakenteensa ja vaikutustapansa perusteella jakaa useisiin eri ryhmiin. IL-21 kuuluu ns. gamma-c-sytokiiniperheen sytokiineihin. Muita tämän sytokiiniperheen jäseniä ovat mm. IL-2, IL-4 ja IL-15. Aktivoidut T-solut tuottavat IL-21:tä ja IL-21 tehostaakin T-solureseptorivälitteistä T-solujen aktivoitumista ja jakautumista. T-solujen lisäksi IL-21 vaikuttaa monin tavoin myös NK-solujen toimintaan. IL-21 mm. voimistaa NK-solujen kykyä tuhota virusten infektoimia soluja. Ensimmäisessä osatyössä tutkittiin miten IL-21 vaikuttaa ihmisen verestä eristettyjen primaari T- ja NK-solujen geeniluentaan ja sitä kautta solujen toimintaan. IL-21 stimulaatio lisää tiettyjen Th1-tyyppisten geenien ilmentymistä sekä NK- että T-soluissa. Yksi näistä aktivoituvista geenistä on interferoni-γ (IFN-γ), jolla on monia tehtäviä sekä

synnynnäisen että adaptiivisen immuniteetin aktivoitumisessa ja ylläpitämisessä. Toisessa osatyössä tutkittiin tarkemmin IL-21:n sekä IL-15:n ja IL-18:n vaikutusta IFN-γ tuotantoon T- ja NK-soluista. Havaitsimme että IL-21 yhdessä IL-15:n ja IL-18:n kanssa lisää merkittävästi IFN-γ tuotantoa T- ja NK-soluista. Analysoimme myös IL-21:n aktivoimia signaalinvälitysreittejä T- ja NK-soluissa ja havaitsimme että IL-21 aktivoi Jak-STAT-signaalireitiltä Jak1/Jak3 sekä STAT3 tyrosiini-fosforylaation mutta ei esimerkiksi NF-κB:tä.

IFN-α/β tuotetaan erityisesti virusinfektion aikana makrofaageista ja dendriittisoluista. IFN-α:lla on suoria antiviraalisia vaikutuksia mutta niiden lisäksi IFN-α/β tehostaa myös NK- ja T-solujen toimintaa. Kolmannessa osatyössä tutkittiin IFN-α/β vaikutusta T-solujen IL-21 tuotantoon sekä T- ja NK-solujen responsiivisyyteen IL-21:lle IFN-α/β esikäsittelyn jälkeen. IFN-α lisää IL-21 tuotantoa aktivoiduista T-soluista. IFN-α stimulaatio edistää STAT-proteiinien sitoutumista IL-21 promoottorille, millä on positiivinen vaikutus IL-21 tuotantoon. Toisaalta havaitsimme myös, että IFN-α stimulaatio vähentää IL-21 reseptorin ilmentymistä NK- ja T-soluissa. IL-21R:n vähentyminen näiden solujen pinnalla johti myös alentuneeseen STAT3 aktivaatioon IL-21 stimulaation jälkeen.

Dendriittisolujen tehtävänä on esitellä antigeeneja epäkypsille T-soluille tunnistusta varten. Dendriittisolujen ottaessa mikrobeja sisäänsä, ne kypsyvät ja vaeltavat paikallisiin imusolmukkeisiin esittelemään antigeeneja T-soluille. Kypsyvien dendriittisolujen pintareseptorien ilmentyminen muuttuu siten, että ne alkavat ilmentää pinnallaan enemmän CD86 (B7-2) ja HLA-II molekyylejä jotka ovat välttämättömiä reseptoreja antigeenin esittelytapahtumassa. Neljännessä osatyössä tutkimme IL-21:n vaikutusta dendriittisolujen kypsymiseen ja aktivoitumiseen. Dendriittisoluja voidaan *in vitro* erilaistaa monosyyteistä. Erilaistuvat dendriittisolut alkavat ilmentää pinnallaan IL-21 reseptoria, mitä puolestaan makrofaagit eivät tee. IL-21 esikäsitellyissä soluissa CD86 ja HLA-II reseptorien ilmentyminen LPS-stimulaation jälkeen on vähentynyt, kuten myös TNF-α, IL-12, CCL5 ja CXCL10 tuotanto. Tulokset viittaavat siihen, että IL-21 estää dendriittisolujen kypsymistä ja aktivoitumista. SOCS-proteiinien on aiemmin raportoitu vähentävän LPS:n aikaansaamaa aktivaatiota. IL-21-stimulaatio lisää SOCS1 ja SOCS3 geenien ilmentymistä dendriittisoluissa ja tämä voi olla ainakin osasyy havaitulle ilmiölle. Kaiken kaikkiaan tutkimuksen tulokset osoittavat, että IL-21:llä on tärkeä rooli sekä synnynnäisen että adaptiivisen immuniteetin säätelyssä.

Avainsanat: immunijärjestelmä, valkosolut, välittäjäaine, geeninsäätely

CONTENTS

Abbreviations **10**

List of original publications **13**

1 Introduction **15**

2 Review of the literature **17**

2.1 CELLS OF THE IMMUNE SYSTEM 17

2.1.1 NK cells 18

2.1.2 T lymphocytes 19

2.1.3 Dendritic cells 20

2.2 CYTOKINES 22

2.3 CYTOKINE-INDUCED SIGNALLING; JAK-STAT PATHWAY 25

2.3.1 Jaks 28

2.3.2 STATs 26

2.3.3 Regulation of Jak-STAT signaling 28

2.4 TH1 CYTOKINES THAT REGULATE NK, T, AND DENDRITIC CELL FUNCTIONS 29

2.4.1 Interferons 29

2.4.2 IL-12 30

2.4.3 IL-18 31

2.4.4 Gamma-c cytokine family 33

3 Aims of the study **37**

4 Materials and methods **38**

4.1 CELL CULTIVATIONS AND CYTOKINE STIMULATIONS 38

4.1.1 Primary T cell culture (I-III) 38

4.1.2 Primary NK cell purification (I-III) 38

4.1.3 Dendritic cell isolation and differentiation (IV) 39

4.1.4 NK-92 cell line (I-III) 39

4.1.5 Cytokine stimulations (I-IV) 39

4.2 RNA ISOLATION AND NORTHERN BLOT ANALYSIS (I-IV) 39

4.3 PROTEIN EXPRESSION ANALYSES 40

4.3.1 Western blotting (III) 40

4.3.2 Immunoprecipitation (I-II 40

4.3.3 Cytokine ELISAs (II, IV) 40

4.3.4 Flow cytometry analysis (IV) 40
4.4 PROTEIN-DNA BINDING STUDIES 41
4.4.1 Electrophoretic mobility shift assay (I-III) 41
4.4.2 Oligonucleotide DNA precipitation (II-III) 41
5 Results and discussion 43
5.1 REGULATION OF IL-21 AND IL-21R EXPRESSION IN NK AND T CELLS 43
5.1.1 IFN-α enhances TCR-mediated activation of IL-21 gene expression in T cells (III) 43
5.1.2 IFN-α down-regulates IL-21R expression in NK and T cells (III) 44
5.2 IL-21-INDUCED GENE EXPRESSION IN NK AND T CELLS 45
5.2.1 IL-21 increases the mRNA expression of Th1 type cytokine receptors (I, II) 45
5.2.2 IL-21 activates Jak/STAT pathway (I, II) 46
5.2.3 IL-21-induced IFN-γ expression (I,II) 47
5.3 IL-21 MODULATES DC FUNCTIONS 49
5.3.1 IL-21R is differentially expressed in human monocyte derived macrophages and DC (IV) 49
5.3.2 DC maturation and cytokine production is inhibited by IL-21(IV) 49
5.3.3 IL-21 induces SOCS gene expression (IV) 50
6 Conclusions 52
7 Acknowledgements 53
8 References 54

ABBREVIATIONS

AP-1	activating protein-1
APC	antigen presenting cell
CD	cluster of differentiation
cDNA	complementary DNA
CTL	cytotoxic T lymphocyte
DC	dendritic cell
dsRNA	double stranded RNA
ELISA	enzyme-linked immmunosorbent assay
EMSA	electrophoretic mobility shift assay
FCS	fetal calf serum
GAS	interferon-γ activated sequence
γc	common γ chain
GF	growth factor
GM-CSF	granulocyte-macrophage colony stimulating factor
GTP	guanidine triphosphate
HLA	human leukocyte antigen
ICAM-1	intercellular adhesion molecule 1
IFN	interferon
Ig	immunoglobulin
IκB	inhibitor of NF-κB
IKK	IκB kinase
IL	interleukin
IRAK	IL-1 receptor-associated kinase
IRF	interferon regulatory factor
ISRE	interferon-stimulated response element

IU	international unit
Jak	janus tyrosine kinase
KIR	killer-cell immunoglobulin-like receptor
LPS	lipopolysaccharide
LTA	lipoteichoic acid
MAPK	mitogen-activated protein kinase
MHC	major histocompatibility complex
MIG	monokine induced by IFN-γ
MyD	myeloid differentiation factor
NCR	natural cytotoxicity receptor
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor kappa B
NK	natural killer
PAGE	polyacrylamide gel electrophoresis
PAMP	pathogen associated molecular pattern
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PIAS	protein inhibitors of activated STATs
PKR	protein kinase R
PMA	phorbol myristate acetate
PRR	pattern recognition receptor
PTP	protein tyrosine phosphatase
RT-PCR	reverse transcriptase polymerase chain reaction
SDS	sodium duodecyl sulphate
SH2	Src homology region 2
SOCS	suppressor of cytokine signalling
ssRNA	single stranded RNA

STAT	signal transducer and activator of transcription
TCR	T cell receptor
TGF	transforming growth factor
Th	T helper cell
TIR	Toll/IL-1 receptor
TLR	toll-like receptor
TNF	tumor necrosis factor
TRAF	TNFR associated factor

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by their Roman numerals:

I Strengell M, Sareneva T, Foster D, Julkunen I, Matikainen S. IL-21 up-regulates the expression of genes associated with innate immunity and Th1 response. Journal of Immunology, 169:3600-3605, 2002

II Strengell M, Matikainen S, Sirén J, Lehtonen A, Foster D, Julkunen I, Sareneva T. IL-21 in synergy with IL-15 or IL-18 enhances IFN-γ production in human NK and T cells. Journal of Immunology, 170:5464-5469, 2003

III Strengell M, Julkunen I, Matikainen S. IFN-α regulates IL-21 and IL-21R expression in human NK and T cells. Journal of Leukocyte Biology, 76:416-422, 2004

IV Strengell M, Lehtonen A, Matikainen S, Julkunen I. IL-21 enhances SOCS gene expression and inhibits LPS-induced cytokine production in human monocyte-derived dendritic cells. Submitted for publication.

1 INTRODUCTION

Human body is continuously in contact with pathogenic microbes from outside environment. These microbes include viruses, bacteria, and protozoa, thus effective antimicrobial defence mechanisms are essential for the survival of an individual. The first defence against harmful microbes is the physical barrier that blocks the invading micro-organisms. The most important part of that is the skin which, when not wounded, is extremely efficient to resist microbes. The second physical barrier is the mucosal tissue, which contains a epithelial tissue and mucosa. If, however, microbes get into the body the immune response is activated and the microbe is normally eradicated.

Immunological system controls the invasion of foreign pathogens to body as well as cleans up the body from malignant cells. Immune system can be divided into two parts; innate and adaptive immunity. The soluble and cellular components of the innate immune system form the first line of defence against invading pathogens. Among the soluble components of the innate immunity are complement, chemokines and cytokines. The cellular components include NK cells that destroy virus-infected cells and macrophages, DCs, and neutrophils that phagocyte and destroy bacteria and other microbes. Often the protection against microbial infection by innate immunity is not complete but the rapid nature of this system provides time and conditions for adaptive immunity to develop.

The development of adaptive immunity requires efficient antigen presentation by APC and recognition of foreign antigens by lymphocytes. This leads to rapid expansion of B and T lymphocytes with specificity for the inducing antigen. The predominant function for B cells is the production of antimicrobial antibodies. The T cell-mediated cellular immunity protects body from intracellular pathogens. T cell receptor recognises foreign antigen processed within a pocket of the major histocompatiblity complex (MHC) molecule on the surface of an infected cell. Cytokines regulate adaptive immune responses by driving T helper 1 or T helper 2 type responses, regulating T cell proliferation, enhancing antigen presentation by macrophages and DC, and by increasing the cytotoxicity of CD8+ T cells.

Cytokines can be divided into different subgroups according to their structure or function. Interleukins are a large group of cytokines, which act, as the name tells, on leukocytes. IL-21 is a novel T cell-derived cytokine that belongs to common-γ-chain (γc) group of cytokines. Along with IL-2, IL-4, IL-7, IL-9, and IL-15 these cytokines utilize γc receptor in their receptor complex and signalling. Although these interleukins are related in their receptor complex, they all have distinct roles in the

regulation of leukocyte functions. Cytokine signaling inside the cells is mediated by complex cascades of signal transduction molecules and transcription factors. Jak-STAT signaling pathway is one of the most important signaling cascades activated by cytokines.

In this thesis work the role of IL-21 in lymphocytes and DC functions was studied. IL-21-inducible genes were identified and the transcription factors activated by IL-21 were analyzed. The regulation of IL-21 gene and its receptor by macrophage/DC-derived IFN-α was also analyzed as well as the regulatory role of IL-21 on maturation and activation of DC.

2 REVIEW OF THE LITERATURE

2.1 Cells of the immune system

The immune system contains a diverse group of cells that are present in the blood and tissues. Despite the diversity of cell types, all cellular elements of blood derive from the same progenitor, hematopoietic stem cells. The development of blood cells occurs in the bone marrow where hematopoietic stem cells first develop to myeloid progenitors, lymphoid progenitors, or erythroblasts. Red blood cells, erythrocytes, differentiate from erythroblasts. White blood cells, leukocytes, on the other hand develop from two lineages (Figure 1). Lymphoid progenitors give rise to B and T lymphocytes, NK cells, and plasmacytoid dendritic cells. Monocytes, which further differentiate into macrophages and myeloid dendritic cells, are obtained from myeloid progenitors. Granulocytes develop also from myeloid progenitors. Granulocyte is a common name for three different cell types, neutrophils, basophils, and eosinophils, which all have cytoplasmic granules that gives them their characteristic phenotype (1).

The cells of the immune system can also be defined based on their functions in the course of immune response. Innate immunity is comprised of phagocytic cells like macrophages, DCs, and neutrofils as well as NK cells. Response by these cells is rapid and does not require specific antigen recognition. Instead, macrophages, DCs, and NK cells recognize conserved microbial components. These components, abundant in pathogens but absent in the host, are recognised by e.g. TLRs, scavenger receptors, and mannose-binding lectins (2). These rapid innate immune responses do not result in the development of immunological memory. The receptors of antigen-specific adaptive immunity, antibodies (in B cells) and T cell receptors (in T cells), 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. The activation of adaptive immunity creates also an immunological memory (1).

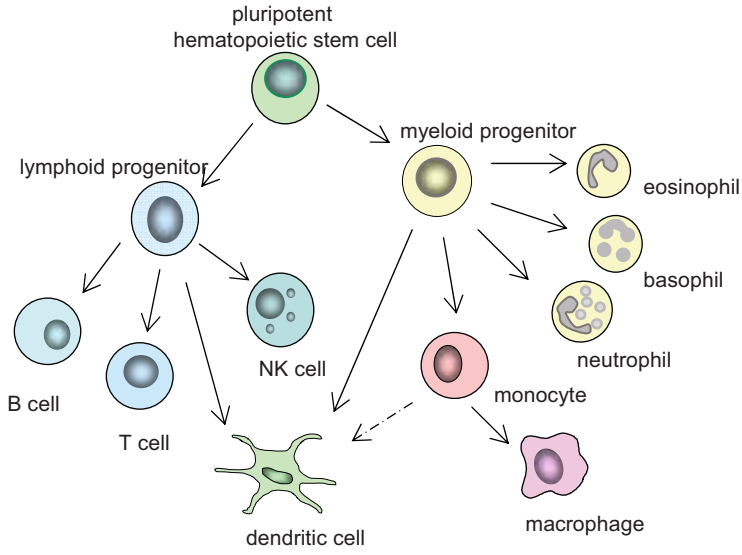

Figure 1. ***Development of human white blood cells. Leukocytes differentiate from pluripotent hematopoietic stem cell and divide to lymphoid and myeloid linages. T and B lymphocytes, NK cells, and plasmacytoid dendritic cells differentiate from lymphoid lineage progenitors. Monocyte/macrophages, granulocytes, and myeloid dendritic cells develop from myeloid lineage progenitors (Adapted from (1).***

2.1.1 NK cells

Natural killer (NK) cells are large granular lymphocytes that comprise about one tenth of all circulating leukocytes. As the name tells their duty is to kill virus-infected or malignant tumor cells (reviewed by (3, 4). NK cells develop within the bone marrow and the development process can be divided into two phases. In the early phase the stem cells develop into NK progenitors (CD34+). NK progenitor cells then differentiate into an NK precursors (CD34+, IL-15R+). In the second phase the precursors develop into mature NK cells (5). Mature NK cells represent two subsets based on their cell-surface density of CD56. The majority (90%) of NK cells are CD56dim. These cells are more cytotoxic but have reduced ability to

produce cytokines compared to CD56bright NK cell subset. CD56bright cells are poorly cytotoxic but they produce high levels of cytokines that regulate NK and T cell functions as well as macrophages and DCs (6, 7).

NK cells express mainly two kinds of receptors. Inhibitory receptors, CD94 and killer immunoglobulin (Ig)-like receptors (KIRs), supress NK cell functions by recognizing MHC class I molecules on the surface of the target cells (8). The expression of MHC class I molecules is down-regulated during virus infection or in tumor cells (9-11). NK cells recognize virus-infected or tumor cells as non-self and eliminate them. On the other hand, NK cells express also stimulatory receptors that activate NK cell cytotoxic response upon ligand binding. These receptors include natural cytotoxicity receptors (NCR) and NKG2D. NKG2D recognize MICA and MICB proteins that are expressed in virus-infected epithelial and endothelial cells or in macrophages (12, 13). NK cell cytotoxic activity against virus-infected cells has been demonstrated in many viral models like in herpesviruses (herpes simplex), orthomyxoviruses (Influenza) and picornaviruses (Coxsackie virus) (reviewed in (14-17).

2.1.2 T lymphocytes

Lymphoid precursors in the bone marrow migrate to thymus in order to differentiate into T lymphocytes. At this time they have neither a complete TCR nor CD4 or CD8 surface molecules. The most important transmembrane structure of T cells is the T cell receptor (TCR). T cells can be divided into two subtypes on the basis of TCR expression. TCRαβ cells recognize peptide antigens associated with self-MHC molecules, whereas TCRγδ cells recognize poorly characterized antigens that are not MHC associated (1). Once committed to TCRαβ lineage, immature thymocytes proceed to the CD4+CD8+ stage where they must choose either CD4+ or CD8+ direction. The exact mechanisms behind the T cell lineage commitment still remain controversial but in general, the cells that recognize antigen in association with class II MHC molecule become CD4+ T cells and those, recognizing antigen with class I MHC molecule become CD8+ T cells. Newly differentiated CD4+ and CD8+ T cells leave thymus as naïve T cells (1, 18).

Each CD4+ T cell has potential to further differentiate into either Th1 or Th2 type CD4+ subset. The antigen stimulus delivered via TCR with costimulatory pathways is essential for the progression of Th differentiation pathway. A number of factors, in addition to antigen stimulus, influence the differentiation process. The most important factor is the cytokine milieu present during differentiation. IL-12, IFN-γ, IL-18, IL-23, and IL-27 significantly drive the differentiation towards Th1 direction (19, 20) whereas IL-4 is the most important cytokine in Th2 differentiation (20-22). CD4+ T helper cells recognize antigens on the surface of an infected cell and help

other immune cells to destroy the invader by secreting cytokines. CD8+ T cells are referred to as cytotoxic T cells as they have the capacity to lyse the antigen presenting target cells. After destroying the microbe-infected cells, most effector T cells die through apoptosis. However, some cells remain as memory T cells, which protect the individual from reinfection (1).

2.1.3 Dendritic cells

Dendritic cells are the most potent antigen presenting cells (APC). They are able to capture and process the antigen and present it to naïve T cells. DCs originate in the bone marrow from a CD34+ progenitor stem cell. Progenitor cells can differentiate into myeloid (CD14+, CD11c-, CD1+) or lymphoid precursor (CD14-, CD11c-, CD123+) cells. Myeloid precursors differentiate into immature myeloid DCs. Lymphoid precursors, on the other hand, differentiate to lymphoid (plasmacytoid) DCs. Myeloid DCs are considered to be responsible for efficient antigen uptake and presentation to T cells whereas lymphoid DCs produce significant amounts of type I interferons (23-26). The migratory features of mDCs and pDCs differ too. mDCs are responsive to several chemokines and thus are rapidly recruited at the site of infection. pDCs, on the other hand, localize predominantly in secondary lymphoid tissues and are responsive only to CXCL12 chemokine (27).

Immature DCs reside in peripheral tissues where they ingest, accumulate and process antigens. Immature DCs predominantly express molecules that enable them to recognize and phagocytose antigens e.g. macrophage mannose receptor, pattern recognition receptors (PRR), reviewed by (28). The most recent PRRs described are the Toll-like receptors (TLR). These are a group of type I transmembrane proteins that recognize pathogen associated molecular patterns (PAMP) on invading bacteria or virus. There are currently ten TLRs described in human. TLR2, TLR4, TLR5, and TLR9 are the receptors associated mostly in recognition of bacterial components like lipopeptides, LPS, flagellins, and bacterial unmethylated CpG rich DNA, respectively. In contrast, TLR3, TLR7, and TLR8 recognize structures (ssRNA and dsRNA) that are usually present in virus infections. Ligand binding to TLR leads to activation of a complex signal cascade that involves several mediators and adapter molecules (Figure 2). TLR signaling leads to activation of many transcription factors; NF-kB, IRF-3, IRF-7, and AP-1, thus regulating various target genes (29-31).

The ingestion and recognition of antigen stimulates DC to mature. This maturation process is accompanied by the upregulation of chemokine receptors and induction of adhesion molecules that allow DCs to migrate to secondary lymphoid tissues (32) and present antigens to naïve T cells. Mature DCs undergo cell surface phenotypic changes, which include the upregulated expression of HLA class II molecules and

costimulatory molecules CD80, CD83, CD86, and CD40, reviewed in (28, 33). HLA class II molecules are needed for antigen presentation to TCR and costimulatory molecules (B7-1, B7-2) for full activation of T cells through CD28 receptor (34). The maturation process also induces cytokine and chemokine production from DC, reviewed in (23, 28).

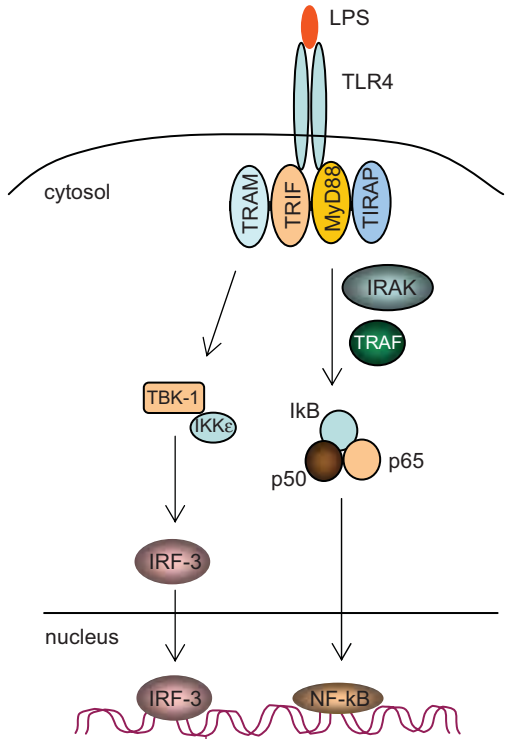

Figure 2. ***Schematic picture of TLR4 signaling. LPS binding to TLR4 induce activation of several adapter molecules (MyD88, TRAM, TRIF, and TIRAP) and kinases (IRAK, TRAF, IKKε, IkB, and TBK-1). This leads to activation of NF-kB and IRF-3 transcription factor, which enter the nucleus and binds to promoter region of LPS-induced target genes to initiate transcription (Adapted from (31)).***

2.2 Cytokines

Cytokines play a crucial role in the development, differentiation, and activation of the cells of the immune system. The proper immune responses require a cross-talk between cells, which involves a variety of cytokines. Cytokines are polypeptides or glycoproteins that mediate signals between the cells of the immune system. Cytokines mediate signals regulating and determining the nature of the immune response and participate in inflammatory processes. Constitutive production of cytokines is usually very low and their production is regulated by various activating stimuli at the level of transcription or translation. Cytokine production is transient and they act by binding to their specific cell surface receptors on target cells. Most cytokine effects result from an altered gene expression pattern in the target cells. Cytokines, having so diverse range of effects, can be classified based on their functions (Table 1). In addition to functional grouping of cytokines, they can be classified based on the structure of their receptors (Table 2). This classification is partially overlapping with the functional grouping of cytokines. Type I cytokine receptor family is the largest family including many important interleukin receptors like IL-2R, IL-4R, IL-12R, and IL-15R. This big group of cytokine receptors can be further divided into subclasses based on the receptor chains shared in these receptors. Class II cytokine receptor family includes receptors for interferons (-α,–β, and -γ) but also some interleukins. TNF receptor family consists of TNF-α and lymphotoxin a and b receptors, Fas ligand, CD40 ligand, and TRAIL. IL-1 receptor family members have similar immunoglobulin superfamily structures in their extracellular domains. The cytosolic region of these receptors is related to Toll-like receptors (TIR domain). Chemokine receptors are a large group of cytokine receptors having classical structure of seven transmembrane domains (35, 36).

Table 1. ***Cytokine families***

Cytokine family	Cytokines	Biological activities
Interleukins (IL)	IL-1-IL-31	Numerous overlapping effects on e.g. regulation of cell growth, proliferation, and differentiation
Interferons (IFN)	IFN-α, –β, –γ, –λ, –ω, –τ	Antiviral, antiproliferative, immunomodulation
Tumor necrosis factors (TNF)	TNF-α, TNF-β	Proinflammatory, apoptosis
Transforming growth factor (TGF)-β family	TGF-β, inhibins/activins, bone morphogenetic proteins	Immunomodulation, morphogenesis, development
Chemokines	C, CC, CXC, and CX3C type chemokines	Chemotaxis
Colony stimulating factors	GM-CSF, G-CSF, M-CSF, IL-3	Growth and differentiation of hematopoietic cells
Growth factors	EPO, TPO, EGF, FGF, PDGF	Growth and differentiation

Adapted from (35, 36)

Table 2. ***Cytokine receptors***

Receptor family	Shared receptor subunits	Representative ligands
Class I cytokine receptors	gp130	IL-6 and IL-11
(Hematopoietin receptors)	shared β-chain	GM-CSF, IL-3, IL-5
	shared γ-chain	IL-2, IL-4, IL-7, IL-9, IL-15, IL-21
	shared α-chain	IL-13, IL-14
	none, specific receptor chains	IL-12, G-CSF, erythropoietin, GH
Class II cytokine receptors		IFN-α/β, IFN-γ
(IFN/IL-10 R family)		IL-10, IL-20, IL-22
TNF receptor family		TNF-α, LT-a, LT-b, Fas ligand
IL-1 receptor family		IL-1a, IL-1b, IL-18
TGF-β receptors		TGF-β
Chemokine receptors	C receptors	C chemokines
	CC receptors	CC chemokines
	CXC receptors	CXC chemokines
	CX3C receptors	CX3C chemokines

Adapted from (35, 36)

2.3 Cytokine-induced signalling; Jak-STAT pathway

The biological functions of cytokines are achieved by binding of cytokines with high affinity to their specific cell-surface receptors. Receptor binding triggers signal transduction cascades that regulate cell activation, differentiation, proliferation, and apoptosis. The Jak/STAT pathway is the principal signalling mechanism for a wide variety of cytokines and growth factors. Intracellular activation occurs when ligand binding induces the multimerization of receptor subunits. In the case of cytokines the receptors are heteromultimers. The multimerization of receptor subunits increases the local concentration of Jaks, which in turn allows Jak proteins to interact and auto- or transphosphorylate each other. Jaks also phosphorylate the receptor chains creating docking sites for Src homology SH2-containing STATs. Jaks subsequently phosphorylate STATs that dimerize through interaction with conserved SH2 domains. Phosphorylated STATs enter the nucleus, where they bind to regulatory sequences on the promoter of the target genes (Figure 3).

2.3.1 Jaks

The Janus kinases (Jaks) are a family of cytoplasmic protein kinases that consists of four members Jak1, Jak2, Jak3, and Tyk2 (37-40). Jak activation stimulates cell proliferation, differentiation, cell migration and apoptosis (41). Jak1, Jak2, and Tyk2 are ubiquitously expressed, while Jak3 is expressed primarily in hematopoietic cells. Jak1 is activated by interferons (IFN-α/β and IFN-γ) and cytokines using receptor subunits gp130 (e.g. IL-6, IL-11) or common γc (e.g. IL-2, IL-4, IL-15) (reviewed in (42, 43). Therefore Jak1 deficiency in mice leads to perinatal lethality with severe neurological defects and impaired immune cell development (44). Jak2 activation is mediated by type I cytokines (GH, EPO, and TPO) and interferons. Also Jak2 deficiency results in embryonal lethality (45). Jak3 plays an important role in hematopoietic cell signalling and it is associated with common γc receptor subunit. It is thus activated by γc-utilizing cytokines IL-2, IL-4, IL-7, IL-9, and IL-15. The absence of Jak3 leads to severe defects in the development and function of the immune system. Jak3 knock-out mice have a decreased number of B cells and they completely lack NK cells (46). T cell numbers are normal but the cells are functionally incompetent (47, 48). Tyk2 is activated primarily by type I IFNs and IL-12 although other cytokines (e.g. IL-6, IL-10, and IL-13) have also been reported to activate Tyk2 (42). IFN and IL-12 signaling is diminished in Tyk2-deficient mice (49).

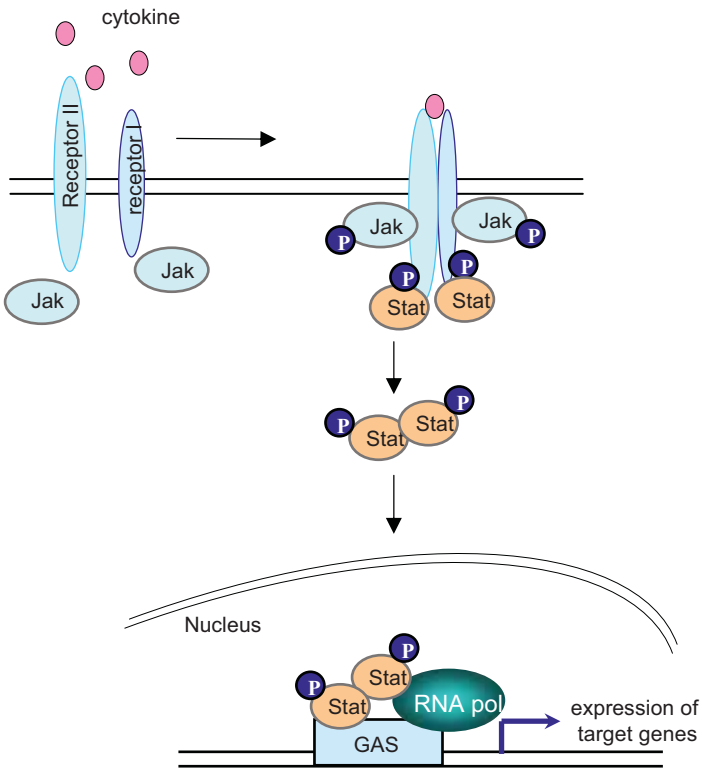

Figure 3. ***Cytokine-induced Jak-STAT signalling. Cytokine binding to its receptor induces receptor heterodimerization and phosphorylation of receptor chains and associated Jak proteins. Jaks phosphorylate STAT proteins that bind with their SH-2 domains to phosphorylated receptor chain. Dimerized STATs translocate into nucleus and bind to GAS element on the promoter region of the target gene and initiate mRNA expression.***

2.3.2 STATs

Seven members of the signal transducer and activator of transcription (STAT) family of transcription factors have been identified in mammals: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6. STATs are cytoplasmic transcription factors involved in cytokine, hormone, and growth factor signaling, for review see (50-52). Ligand cytokine binding leads to receptor homo- or heterodimerization of the receptor chains. After this Jak kinases are activated by phosphorylation of tyrosine residues in the carboxy terminal and then STATs are

phosphorylated by Jaks. The modified STATs are released from the cytoplasmic region of the receptor subunits to form homodimers or heterodimers. Dimers are formed thourgh interaction between the phosphotyrosine of one STAT and the SH2 domain of another. Following dimerization, STATs rapidly translocate to the nucleus and interact with specific regulatory elements to induce target gene expression, reviewed by (50, 51, 53).

STAT1 is involved in IFN signaling. STAT1 deficiency results in susceptibility to viral infections and intracellular pathogens. STAT1 mediates the anti-viral and inflammatory effects of IFNs through the induction of genes such as MHC, costimulatory molecules, chemokines, complement genes, and IRF-1 among others (54, 55). STAT1 also has a role in suppressing autoimmune disorders and it is important in host anti-tumor responses. STAT2 is activated by IFNα/β and IFN-γ. STAT2 is necessary for antiviral responses of IFN-α/β (56). STAT2 together with STAT1 form a heterodimer that associates with a member of the IFN regulatory factor (IRF-9), p48. They form ISGF3 complex that translocates to the nucleus to initiate gene transcription by binding to interferon-stimulated response elements (ISRE) (reviewed in (57, 58).

STAT3 is activated by many cytokines. Therefore it is not surprising that mice lacking STAT3 exhibit perinatal lethality (59). The conditional knockout approach has revealed specific defects in STAT3-deficient T cells. STAT3 is required for optimal IL-2-induced T cell proliferative responses by upregulating the expression of IL-2Rα. STAT3 deficient cells show impaired IL-2-induced IL-2Rα expression and defect in IL-2-induced proliferation, similar to that observed in STAT5-deficient mice (60). STAT3 is also important in IL-6-mediated suppression of apoptosis in T cells. IL-6 responses in STAT3 deficient T cells are impaired (61). STAT3 deficiency in myeloid cells results in hyperactivation of macrophages and neutrophils, marked increases in inflammatory cytokine production, and inflammatory bowel disease demonstrating the immuno-suppressive and anti-inflammatory function of STAT3 in the myeloid lineage (62). IL-10 responsiveness is completely abolished in STAT3 deficient macrophages (63).

STAT4 is activated by IL-12, IL-23, and IFN-α (64-66). IL-12 is a major regulator of Th1 cell differentiation and its functions are mediated through STAT4 activation (67-69). STAT4 deficient mice exhibit impaired Th1 differentation, IFN-γ production, and cell-mediated immune responses. CD4+ T cells from these mice were incapable of producing high levels of IFN-γ in response to IL-12 priming. In addition, IL-12 stimulation did not augment NK cell cytotoxicity in these mice (70).

STAT5a and STAT5b are closely related STAT proteins that have overlapping but somewhat distinct functions (71, 72). STAT5a and STAT5b are activated by IL-2

family of cytokines (those using γc, excluding IL-4, IL-13, and IL-21) (73). STAT5a deficiency causes deficit in prolactin signaling. STAT5b deficiency leads to impaired growth hormone secretion. Deletion of STAT5a and STAT5b cause defects in immune responses due to inadequate IL-2-induced responses. Double knockout mice have no NK cells, T cells are not able to proliferate in response to T cell receptor ligation and IL-2 receptor activation (72, 73).

STAT6 is activated by IL-4 and IL-13. STAT6 target genes include immunoglobulin heavy chain ε, CD23, MHC-II, GATA-3, and c-maf. STAT6-deficiency causes defects in T cell compartment, specifically in IL-4-induced Th2 differentiation and IL-4-stimulated increases in expression of CD23, MHC class II molecules and IL-4Rα. STAT6-deficiency also results in diminished IgE production (74, 75)

2.3.3 Regulation of Jak-STAT signaling

Cytokine stimulus results in transient activation of Jak-STAT signaling pathway, which usually peaks 5-30 min after stimulation. However, for maintaining homeostasis or preventing chronic inflammation or autoimmunity, Jak-STAT signaling is also negatively regulated by multiple mechanisms. Jak and STAT proteins are substrates for several protein tyrosine phosphatases. The SH2 domain-containing tyrosine phosphatases SHP-1 and SHP-2 downregulate signaling by dephosphorylating Jaks and STATs. PIAS (protein inhibitors of activated STATs) proteins are able to block STAT DNA binding. The regulatory role of PIAS in STAT signaling has been demonstrated in Drosophila and the function of mammalian PIAS remains unclear, reviewed by (52, 76).

SOCS proteins are a family of eight members (SOCS1-7 and CIS). These proteins have a SH2 domain and a SOCS box at the C-terminus. SOCS1, SOCS2, SOCS3, and CIS have been shown to inhibit cytokine-induced Jak-STAT signaling. SOCS1 downregulates the signaling of many cytokines including IL-6, IL-4 and IFNs. SOCS1 inhibits signal transduction by inhibiting the kinase activity of Jaks. SOCS3 regulates the signal transduction of several same cytokines as SOCS1 but additionally IL-2 and IL-3. SOCS3 interacts with Jaks but with a much weaker affinity as compared to SOCS1. Instead, SOCS3 binds to SHP-2 binding sites on the activated cytokine receptors. SOCS3 thus blocks the binding sites for STATs and may also have inhibitory effects on Jaks. The same mechanism is propably used by SOCS2 as well. CIS binds to tyrosine phosphorylated receptor subunit by blocking STAT5 binding sites. CIS was first characterized as a classical feedback modulator of STAT5-mediated signaling, for review see (77-79).

2.4 Th1 cytokines that regulate NK, T, and dendritic cell functions

2.4.1 Interferons

INFs are molecules that are secreted and used in the communication between the cells of the immune system. Interferons are named by their ability to interfere viral replication and infection. Interferons can be divided in two groups; Type I interferons are interferon-α, –β, –δ, –κ, –ω, –τ, and two recently characterized lambda-interferons IL-28 and IL-29 (80). Type II interferon group contains only one member; interferon-γ. There are at least 15 genes coding for IFN-α but only a single gene for IFN-β in the human genome. In human tissues IFNs are expressed in a low basal level even in the absence of specific inducer. Generally microbial infections (viruses, bacteria), dsRNA, CpG-DNA, and other microbial components induce IFN production. Interferons are produced by many cell types but macrophages and dendritic cells are considered as the main producers of interferons (81).

IFN receptors are composed of two receptor chains (IFN-α/βR1, IFN-α/βR2, IFN-γR1, and IFN-γR2) that dimerize by binding with their ligands. Binding of IFN-α/β induces Jak1 and Tyk2 activation, which leads to tyrosine phosphorylation of intracellular domains of each receptor chain and STAT proteins. Dimerized STAT proteins translocate into nucleus and bind to ISRE and GAS regulatory elements in the promoters of IFN-activated genes (57, 82, 83).

IFN-α/β induces several antiviral proteins, including protein kinase R (PKR), oligoadenylate synthetases (OAS), and Mx proteins. Activation of PKR leads to general inhibition of protein synthesis, which prevents viral protein synthesis and replication (84). OAS activation leads to degradation of host cell mRNAs (85). Mx proteins are GTPases that inhibit viral replication by inhibiting trafficking of viral particles (85). IFN-α/β promotes innate and adaptive immune responses by activating the expression of several cytokines and their receptor genes and having a number of immunomodulatory effects. IFN–α-induced IL-15 drives the proliferation and maturation of NK cells (86, 87). IFN-α also enhances the cytotoxic functions of NK cells (reviewed in (14)) and enhances IL-2Rα, IL-12R, IL-18R, and IFN-γ gene expression in NK cells (88-91). IFN-α enhances antigen processing and presentation by stimulating the expression of MHC class I molecules and costimulatory molecules ICAM-1, B7-1, and B7-2 in APC. In addition, IFN-α induces maturation and activation of DCs (92). IFN-α/β promotes the survival of activated T cells (93) and enhances virus-specific CTL functions. IFN-α-induced IL-15 stimulates the proliferation of memory T cells (94, 95).

Type II interferon, IFN-γ, is produced mainly by NK and T cells. Recent studies show that B cells and APCs can also secrete minor amounts of IFN–γ (96, 97). IFN-γ production is controlled by APC-derived cytokines (reviewed in (98). The most important is IL-12, which is secreted by macrophages after microbial stimulus. Other APC-derived cytokines TNF-α, IL-15, IL-18 as well as type I interferons (in synergy with IL-12) stimulate IFN-γ production from NK and T cells (67, 99, 100). Combinations of these cytokines synergistically enhance the production of IFN-γ. IFN-γ production is inhibited by IL-4, IL-10 and TGF-β (69).

IFN-γ receptor is composed of two receptor chains, IFN-γR1 and IFN-γR2 and ligand binding induces Jak1-Jak2 phosphorylation and activation. Jaks phosphorylate receptor chains to form docking sites for the SH2 domain of STAT1 protein. STAT1 proteins then dimerize and enter the nucleus. STATs bind to GAS elements to initiate or suppress transcription of IFN-γ-regulated genes (reviewed in (57, 101, 102). IFN-γ-regulated genes include several IRFs (IRF-1, -4, -8, -9), ICAM, MIG, and iNOS. IFN-γ signaling is mainly down-regulated by SOCS-1, which is one of the most inducible targets of IFN-γ (103, 104). In addition, nuclear dephosphorylation of STAT1 may control IFN-γ signaling.

IFN-γ has multiple immunomodulatory effects, many of which overlap with type I interferons. IFN-γ promotes innate immune responses by activating phagocytosis and NK cell effector functions (105). IFN-γ is the major product of Th1 cells and it further directs immune response toward Th1 phenotype by enhancing the microbe-stimulated expression of Th1 cytokine IL-12 in macrophages and DCs. On the other hand, IFN-γ inhibits IL-4 secretion by Th2 cells. IFN-γ induces the expression of several chemokines, including CCL2, CCL3, CCL4, CCL5, CXCL9, and CXCL10. IFN-γ promotes antigen presentation by upregulating the expression of MHC class I and class II molecules and B7.2 costimulatory molecule on B cells, DC and monocyte/macrophages. This further enhances CD4+ differentiation. Like type I interferons, IFN-γ also has direct antiviral effects.

2.4.2 IL-12

IL-12 family consists of three heterodimeric cytokines, IL-12 (p35, p40), IL-23 (p19, p40), and IL-27 (EBI-3, p28) (106). All these cytokines are produced by hematopoietic and endothelial cells (107, 108). The main producers of these cytokines are monocytes, macrophages and DC. The gene expression of IL-12 subunits is regulated independently. Most cells express p35 constitutively but p40 gene is expressed only in APCs. In contrast to other cell types that produce p35 spontaneously, monocytes, macrophages, and DCs require additional stimuli to produce p35. This provides a mechanism to prevent uncontrolled IL-12 production:

p40 expression determines the ability of a given cell type to produce IL-12 but the amount of produced cytokine is determined by the expression of p35 (109, 110). A variety of pathogenic organisms, including bacteria, viruses, parasites, and fungi, induce high levels of IL-12 production. Microbial products such as LPS, lipoteichoic acid (LTA), peptidoglycan and bacterial CpG DNA induce production of IL-12 via TLR signaling. IL-12 is also produced in a T cell-dependent manner through the engagement of CD40 on APC with its receptor CD40L on T cells.

IL-12R consists of two receptor chains IL-12Rβ1 and IL-12Rβ2 and they are expressed in NK and T cells and DCs (111). p40 interacts with the β1 subunit and p35 with the β2 subunit. The β2 subunit is the signal transducing chain of the receptor while β1 subunit is involved in ligand binding. IL-12R expression is highly regulated on T cells so that it is not expressed in resting T cells but the expression of IL-12R is induced by T cell activation (112). In different Th type cells the IL-12R is differentially expressed. It is expressed at high level in Th1 cells and IFN-γ stimulus enhances IL-12R expression in these cells. In contrast, Th2 type cytokine IL-4 inhibits IL-12Rβ2 expression and in Th2 cells IL-12R is absent (112). Like other cytokines, IL-12 utilizes Jak-STAT pathway in its signaling. Jak2 and Tyk2 are activated by IL-12 binding to its receptor. STAT1, STAT3, and STAT4 are reported to be activated by IL-12 but STAT4 is the most important player in IL-12 signaling. IL-12-mediated signaling and induction of IFN-γ requires the activation of STAT4 (68, 113, 114). STAT4 knockout mice show impaired IFN-γ production and Th1 polarization (70).

IL-12 influences DC, NK and T cell biology in many ways (108). IL-12 initially induces IFN-γ production in NK and T cells. IL-12-induced IFN-γ production is synergistically enhanced by IL-18 (and IFNα/β) (115). In addition, IL-12 enhances IL-18R expression in NK and T cells. IL-12 regulates adaptive immunity. Th1 cells produce IFN-γ and promote cell-mediated immunity, which is essential for the response against intra-cellular pathogens like viruses. IL-12 is the main cytokine that regulates Th1 differentiation and has a number of important actions that promote cell-mediated immunity. IL-12 induces T cell proliferation; it preferentially acts on naïve CD4+ T cells to induce their differentiation and expansion. IL-12 and IFN-γ antagonize Th2 differentiation and the production of IL-4, IL-5, and IL-13. In addition, IL-12 especially in combination with IL-18 induces IFN-γ production in DC (96, 116).

2.4.3 IL-18

IL-18 is a member of the IL-1 cytokine superfamily and it was originally identified as an IFN-γ-inducing factor (117). This capability of IL-18 to induce IFN-γ in the

presence of IL-12 separates IL-18 saliently from IL-1. IL-18 gene contains two distinct promoter elements. It has been reported that IL-18 promoter contains binding sites for at least NF-kB, IRF-8, PU.1, and AP-1 transcription factors. IL-18 is produced in the cells in a proIL-18 form, which is biologically inactive (118). This pro-form has to be cleaved into mature IL-18 before secretion. Caspase-1 is the most important enzyme in IL-18 processing but propably some other enzymes, like metalloproteinases (MMP-2, -3, -4) and proteinase 3 can cleave pro-IL-18 into its mature form (119, 120). Anyhow, only the cells with appropriate processing machinery are able to produce biologically active form of IL-18. The principal source of IL-18 is macrophages but IL-18 is produced by wide range of cell types such as DC, keratinocytes, Langerhans cells, and intestine epithelial cells (121, 122). The production of biologically active IL-18 is stimulated by several viruses and bacteria. In addition, microbial components like LPS induce IL-18 secretion from macrophages and DC.

IL-18R belongs to the IL-1R family and therefore these cytokines utilize similar signaling pathways (123, 124). IL-18 binds first to IL-18Rα, after which the second chain, IL-18Rβ, binds to the receptor complex. After formation of the receptor-ligand complex, a cytosolic protein MyD88 is recruited. MyD88 has many characteristics of cytoplasmic domains of receptors, but it lacks an extracellular or transmembrane structures. MyD88 deficient mice do not respond to IL-1 or IL-18 (125). They also have diminished cytokine responses and Th1 cells in these mice are not responsive to IL-18-induced activation of NF-kB and c-jun (125). Thus MyD88 is essential for IL-18 (and IL-1) signaling. MyD88 acts as an adaptor molecule and recruites several kinases like IRAK, TRAF-6, and IKKα/β to mediate IL-18 signaling. This cascade leads to the activation of NF-kB and MAPK p38 and activates transcription.

IFN-γ is one of the most important target genes of IL-18. In general, IL-18 alone does not induce IFN-γ production but combined stimulation of IL-18 and IL-12 or IFN-α induces remarkably high levels of IFN-γ in CD4+, CD8+ T cells and NK cells (89, 115, 117, 126-128). Enhancement of IFN-γ production strongly promotes the development of Th1 type immune responses (129). However, recent studies have shown that IL-18 is able to promote Th2 type responses (126, 129). IL-18 stimulation induces Th2 type cytokines IL-4 and IL-13 in NK cells, naïve T cells, basophils, and mast cells in the presence of IL-2 or IL-3 (130). IL-18 may also act as an autocrine activator of macrophages and DCs. At least murine macrophages and DCs produce IFN-γ when stimulated with IL-18 and IL-12 (96, 131). IL-18 augments the cytolytic activity of NK cells (117, 132) and NK cells from IL-18 deficient mice show impaired killing functions (133). IL-18 also activates CD8+ T cell cytotoxic activity (134). In addition to IFN-γ, IL-18 stimulates the production of

a large number of other cytokines and chemokines, including IL-1α, IL-1β, IL-6, IL-8 , IL-13, GM-CSF, and TNF-α (135-138).

2.4.4 Gamma-c cytokine family

Gamma c cytokine receptor chain (γc) is constitutively expressed in lymphocytes. In monocyte lineage cells γc is expressed at low level but it can be induced by IL-2 and INF-γ stimulation. γc cytokine family members, IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21, all share γc in their receptor complex (139-141). In addition, these cytokines have their own specific receptor chains that give the specificity for each cytokine and regulate their functions. Individual specific receptor chains also show homology to each other and for example IL-2Rβ, IL-15Rα, and IL-21R are structurally related to each other.

IL-2 and IL-15

IL-2 and IL-15 are functionally related cytokines that share a receptor chain IL-2Rβ in their receptor complex (142). In addition, the specific cytokine receptor chains IL-15Rα and IL-2Rα have a strong sequence homology. Both these receptors are widely distributed in different cell types. IL-2Rα and IL-15Rα are expressed e.g. in T and B lymphocytes, NK cells, and monocyte/macrophages (140, 143). IL-2 and IL-15 share a receptor chain, which means that they utilize similar signaling pathways. IL-2/IL-15Rβ receptor binding leads to the activation of Jak1 and γc associates with Jak3 activation (144). The activation of Jak kinases leads to phosphorylation of the receptor chains and further activates signaling cascades including phosphatidylinositol-3 kinase (PI3K) and STATs (mainly STAT5), which turns on the transcription of certain IL-2- and/or IL-15-inducible genes (145).

IL-2 is primarily expressed by CD4+ T cells after TCR/CD3 stimulation. IL-2 production can be induced also by PMA/ionomycin stimulation. Anti-CD28 stimulation enhances TCR-mediated induction of IL-2 production. IL-2 promoter contains many binding sites for stimulation dependent transcription factors like NF-AT, AP-1, and NF-kB (146). The main biological function of IL-2 is the stimulation of T cell proliferation (147). DC from IL-2-/- mice show reduced capacity to induce T cell proliferation . IL-2 also plays a key role in Fas-induced cell death of CD4+ T cells in response to antigen restimulation. This is a critical phenomenon in peripherial tolerance of auto-reactive T cells (148). Further, IL-2 promotes the growth and differentiation of antigen-activated B cells and augments the cytolytic activity of NK cells. In monocytes IL-2 induce GM-CSF, IL-1b, and IL-6 production.

IL-15Rα has a wide tissue distribution, which leads to broad range of cellular targets for IL-15. IL-15 is produced primarily by activated monocyte/macrophages and DCs especially after CD40L-mediated stimulation. Regulation of IL-15 gene expression is suggested to be mostly regulated post-transcriptionally i.e. at the level of mRNA translation and intracellular processing and secretion of the protein. IL-15 acts to extend the survival of lymphocytes (149, 150) by acting as a growh factor and by inhibiting IL-2-mediated antigen-induced cell death of CD4+ T cells (149, 151). IL-15 thus plays a role in the promotion of long-term maintenance of antigen-expressing CD4+ T cells (152). IL-15 acts as a growth factor especially for NK cells (153-155). IL-15-deficient mice show marked reduction in the numbers of NK cells and NKT cells (156). Furthermore they show a reduction in the number of memory CD8+ T cells (157). IL-15Rα-deficient mice also showed a profound defect in the function of NK cells and memory CD8+ T cells. In contrast, in IL-15 transgenic mice the IL-2-induced cell death of T cells was inhibited and these mice had increased numbers of NK cells. IL-2 and IL-15 play opposite roles in the control of the homeostasis of CD8+ memory phonotype T cells. Zhang and coworkers (94) have demonstrated that unlike IL-2, IL-15 provides potent and selective stimulation of memory CD8+ T cells and that the proliferation of CD8+ T cells of memory phenotype is stimulated by IL-15 but it is inhibited by IL-2 (157, 158).

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The most recently identified member of gamma-c-cytokine family is IL-21 (159). IL-21 is produced by anti-CD3 and anti-CD28 or phorbol myristate acetate (PMA) and ionomycin activated CD4+ T cells (159). These cells are sofar the only reported source of IL-21. More detailed analysis revealed that in mice IL-21 is selectively produced by Th2 cells. The regulatory mechanisms of IL-21 production are still largely unclear but some data has been acquired from mouse experiments. Recent studies have revealed that NFATc2 and T-bet transcription factors contribute to IL-21 production in murine Th2 cells (160). It has been shown that IL-21 promoter contains three NFAT binding sites, which are important for TCR-stimulated IL-21 promoter activity. NFATc1 and NFATc2 are expressed in both Th1 and Th2 cells and are essential for cytokine gene expression. T cells deficient in NFATc1 and NFATc2 are severly impaired in the production for Th1 and Th2 cell-specific cytokines (161). NFATc2 is considered to be a positive regulator for Th2 cytokines whereas T-bet is a Th1 associated transcription factor, which induces IFN-γ and IL-12R expression in Th1 cells. IL-21 promoter analysis by Mehta and coworkers revealed that NFATc2 activates IL-21 transcription in murine Th2 cells whereas T-bet represses IL-21 expression in murine Th1 cells (160). In contrast, a recent paper by Kim et al. shows that calcium signal leading to NFAT binding to IL-21 promoter is sufficient to activate IL-21 gene expression in murine spleen CD4+ T cells and

that IL-21 expression remains strong in NFATc2 knockout T cells (Kim 2005). The explanation for this discrepancy is still missing.

IL-21R is expressed on many cell types; T, B, NK cells and DCs (159, 162). IL-21R is a heterodimer that consists of γc and IL-21R chain. It has been shown that IL-21R chain alone is able to bind IL-21 but without γc, intracellular signal was not mediated. Thus, γc is required for transducing IL-21 signaling. IL-21R chain shows a high sequence homology to IL-2Rβ and IL-15Rα chains. However, the signal mediated through IL-21R leads to diverse effects from IL-2R or IL-15R.

In T cells IL-21 promotes anti-CD3 and antigen-induced proliferation. IL-21 regulates CD8+ T cell expansion and effector functions, primarily in synergy with IL-15 (163). IL-21 enhances the activation and clonal expansion of antigen-specific CD8+ T cells. Unlike IL-2, which induces activation-induced cell death, IL-21 sustains T cell numbers and promotes their survival (164). On the other hand IL-21 inhibits the differentiation and development of anti-CD3 independent CD8 memory T cells. IL-21 thus regulates T cell functions towards Th1 type response by enhancing antigen-induced proliferation of T cells and promoting cytotoxic T cell responses. The study by Wurster (165) however shows that Th1 type response is also limited by IL-21. They reported that IL-21 inhibits naïve T cell polarization to IFN-γ producing Th1 cells.

IL-21 regulates NK cell functions in many ways. Activated NK cell functions are inhibited by IL-21 as IL-21 reduces IL-15-induced proliferation of NK cells (166). This results in the reduction in the number of activated NK cells rapidly. In addition, IL-21 enhances the cytotoxic activity of NK cells but also promotes the apoptosis of activated NK cells. All this limits the innate immune response in the site of infection. IL-21 has no effect on resting NK cells and thus IL-21 does not seem to play a role in NK cell development. The data from IL-21R knock-out mice also supports this assumption. IL-21R-/- mice have normal numbers and normally functioning NK cells (166, 167). Further, IL-21 stimulates the proliferation of NK precursors (168). IL-21 also upregulates the expression of CD16 surface marker on NK cells. CD16 is an IgG receptor that is involved in antibody-mediated cytotoxicity (168). Overall IL-21 enhances NK cell-mediated innate immune responses by enhancing the proliferation of precursor cells and enhancing their cytotoxic activity. At the same time IL-21 limits the response by inducing apoptosis in already activated NK cells and inhibiting IL-15-induced proliferation, which reduces the number of NK cells.

In B lymphocytes IL-21 increases the proliferation of CD40-activated B cells but reduces the proliferation of IL-4-activated B cells (169). IL-21 also promotes the production of IgG antibodies instead of IgE antibodies, which also promotes the Th1

type response in B cells (167, 170). IL-21 reduces the expression of antiapoptotic genes Bcl-2 and Bcl-x in B cells, which indirectly promotes the apoptosis of these cells (171).

Sofar the only data concerning the role of IL-21 in regulation of antigen presenting cells is from mouse studies. Brandt and coworkers have shown that IL-21 inhibits the maturation and proliferation of murine bone marrow derived dendritic cells. LPS-induced maturation was reduced in these cells and dendritic cells differentiated in the presence of IL-21 showed a reduced ability to induce antigen specific T cell responses.

3 AIMS OF THE STUDY

Cytokines are essential part of immune system mediating signals between immune cells. New cytokines have been characterized recently and this provides new insights for the function of whole immune system network. IL-21 is one of the recently characterized cytokines and in many respects its role in immune system is unclear.

The principal aim of this study was to clarify the role of IL-21 in the activation of innate and adaptive immune responses. In the present study the effect of IL-21 in macrophages, DC, NK, and T cells was studied.

The specific aims were:

1) To study IL-21R and IL-21 expression in NK and T cells

2) To study the effect of IL-21 on NK and T cell regulatory genes

3) To characterize signalling pathways activated by IL-21, especially in relation to the Jak-STAT pathway

4) To study the role of IL-21 in human macrophages and DCs

4 MATERIALS AND METHODS

Human primary cells for experiments were obtained from two to four individual blood donors and stimulated separately to control the interindividual variation. The samples for protein and RNA analysis were pooled, whereas in ELISA and flow cytometry assays the samples were analysed separately. Detailed reaction conditions and reagent concentrations are described in the original communications (I-IV).

4.1 Cell cultivations and cytokine stimulations

Human primary T, NK, and DC were isolated from leukocyte-rich buffy coats, which were obtained from healthy blood donors (Finnish Red Cross Blood Transfusion Service, Helsinki, Finland). Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation using Ficoll-Paque (Amersham Pharmacia Biotech) and T, NK, and DC were further purified as described below and in original communications.

4.1.1 Primary T cell culture (I-III)

Adherent cells from PBMCs were removed by plating the cells on plastic plates for one hour. Non-adherent cells were purified by nylon wool columns to receive enriched T cell population. Enriched population of naïve T cells were either used directly in stimulation experiments or activated with 0.5 μg/ml anti-CD3 and 0.5 μg/ml anti-CD28 monoclonal antibodies (R&D Systems) and cultured in RPMI 1640 medium supplemented with 10% FCS (Integro), antibiotics, and 100 IU/ml human recombinant IL-2 (R&D Systems) for 5-6 days. T cells were then expanded for 5-6 days in RPMI supplemented with 100 IU/ml of human rIL-2.

4.1.2 Primary NK cell purification (I-III)

NK cells were purified from non-adherent PBMCs by nylon wool columns and two-step density gradient centrifugation by Percoll (Amersham Pharmacia Biotech) followed by a negative selection using magnetic beads coated with anti-CD3, anti-CD14, and anti-CD19 antibodies (Dynal Biotech).

4.1.3 Dendritic cell isolation and differentiation (IV)

Mononuclear cells from Ficoll-Paque gradient were further centrifuged in Percoll gradient as described earlier (172). After centrifugation monocytes remaining on top of the gradient were collected and purified with magnetic beads coated with anti-CD3 and anti-CD19 antibodies in order to remove any remaining T and B cells. Purified monocytes were then plated on plastic cell culture plates for one hour. Non-adherent cells were washed away with sterile PBS and monocytes were differentiated to dendritic cells in RPMI media supplemented with 10% FCS, antibiotics, GM-CSF 10 ng/ml, and rIL-4 20 ng/ml. Fresh medium was added every two days and the cells were differentiated for six days.

4.1.4 NK-92 cell line (I-III)

Human NK-92 cells line (173) (ATCC) was maintained in continous culture in MEM Alpha Medium (Gibco Life Technologies) supplemented with 12% horse serum (Gibco), 12% FCS, 0.2 mM i-inositol, 20 mM folic acid, 40 mM 2-ME, 2 mM L-glutamine, antibiotics, and 100 IU/ml rIL-2

4.1.5 Cytokine stimulations (I-IV)

Recombinant human IL-21 was kindly provided by Dr. Don Foster (Zymogenetics), Recombinant human IL-12, IL-15, and IL-18 were purchased from R&D Systems. Highly purified human leukocyte IFN-α (13 x 10^6 IU/ml) and IFN-γ were provided by Dr. Hannele Tölö (Finnish Red Cross Blood Transfusion Service). Recombinant human IFN-α2b (Intron A, 10 x 10^6 IU/ml) and IFN-β (Betaferon, 8 x 10^6 IU/ml) were purchased from Schering-Plough. The cytokine concentrations used were as follows, unless otherwise indicated: IL-15 5 ng/ml, IL-18 10 ng/ml, IL-12 5 ng/ml, IL-21 10 ng/ml, IFN-α, IFN-α2b, and IFN-β 100 IU/ml, and IFN-γ 100 IU/ml.

4.2 RNA isolation and Northern blot analysis (I-IV)

Northern blot analysis was used to analyse the gene expression in cytokine- or TLR-stimulated cells. Stimulated and control cells were harvested at different time points after cytokine stimulation. Total cellular RNA was isolated either by the guanidium isothiocyanate/cesium chloride method, or by using RNA purification kit (Qiagen).

Equal amounts of RNA were size-fractionated on 1% formaldehyde-agarose gels, transferred to nylon membranes (Hybond, Amersham Biosciences), and hybridized with cDNA probes. Ethidium bromide staining of ribosomal RNA or probing with

constant housekeeping gene probes β-actin or GAPDH were used to ensure equal RNA loading.

4.3 Protein expression analyses

4.3.1 Western blotting (III)

Total proteins from cytokine-stimulated and control cells were separated on SDS-PAGE and transferred electrophoretically onto membranes. The membranes were probed with antiphospho-STAT1, antiphospho-STAT3, anti-STAT1, and anti-STAT3 antibodies (Santa Cruz Biotechnology). The specific bands were visualized by the ECL chemiluminescence system (Amersham).

4.3.2 Immunoprecipitation (I-II)

Cleared cell lysates were immunoprecipitated on ice with anti-Jak 1, anti-Jak3, anti-STAT3, anti-STAT5, or anti-phosphotyrosine antibodies. The immunoprecipitated proteins were separated on SDS-PAGE, transferred onto membranes and analysed by anti-phosphotyrosine or anti-STAT1 and anti-STAT4 antibodies.

4.3.3 Cytokine ELISAs (II, IV)

Supernatants from cell stimulation experiments (II) or from the cytokine-priming-LPS-stimulation experiments (IV) were collected. ELISAs for detecting IFN-γ and IL-12 p70 levels were performed using kits from Diaclone. TNF-α, CXCL10, and CCL5 determinations were carried out with matched antibody pairs and ELISA standards (Pharmingen) according to the procedure recommended by the manufacturer.

4.3.4 Flow cytometry analysis (IV)

DC were primed with IL-21 or IFN-γ for 18h followed by stimulation with LPS. Control cells were left without cytokine or LPS stimulation. Stimulated and control cells were harvested and fixed with 1% paraformaldehyde. The fixed cells were stained with fluorescein isothiocyanate (FITC)-conjucated anti-HLA-DR or phycoerythrin (PE)-conjucated anti-CD86 antibodies (Caltaq Laboratories) and analysed with FACScan using Cellquest software (Becton Dickinson).

4.4 Protein-DNA binding studies

4.4.1 Electrophoretic mobility shift assay (I-III)

Nuclear extracts from cytokine-stimulated and untreated cells were made by lysing the cells and isolating the nuclei. The isolated nuclei were then lysed, nuclear protein extract was obtained, and nuclear protein/DNA binding reactions were performed. The probe oligonucleotides (listed in Table 3) were labelled with (α-^{32}P) dATP (3000 Ci/mmol, Amersham) using Klenow polymerase. The binding reaction was done at room temperature for 30 min. Samples were analysed by electrophoresis on non-denaturing low-ionic strength polyacrylamide gels. The gels were dried and visualised by autoradiograpgy.

4.4.2 Oligonucleotide DNA precipitation (II-III)

Cleared whole cell lysates from cytokine-stimulated and control cells were incubated with streptavidin-agarose beads coupled to 5'-biotinylated oligonucleotides (listed in Table 3). The binding reactions were performed for 2 h at +4^{o}C. The oligonucleotide-bound proteins were released in SDS sample buffer, separated on SDS-PAGE, transferred onto membranes, and probed by anti-STAT antibodies to detect the STAT-proteins that were bound to different DNA elements.

Table 3. ***Oligonucleotides used in Electrophoretic mobility shift assays and Oligonucleotide DNA precipitation assays***

DNA element	**Sequence (5' →3')**	**Ref.**
IL-2Rα GAS	GATCTTTCTTCTAGGAAGTACCAAACATTTCTGATAATAGAA	I
MyD88 GAS	GATCGGAGCTTCTCGGAAAGCGAAAGAAGGA	I
SIE	GATCGATCTAGGGATTTCCGGGAAATGAAGCT	I
CIS GAS1	CCCCGTTTTCCTGGAAAGTTTTGGAAATCTGT	III
IFN-γ promoter GAS	GGATCCAGTCCTTGAATGGTGTGAAGTAAAAGTGCCTTCAAAGAATCCC	II
Mutated IFN-γ GAS	GGATCCAGTCCTTGAATGGTGTGAAGTAAAAGTGCCT*CAAAcAATCCC	II
IFN-γ 1st intron GAS	GGATCCTGTTTAAAAATTTTAAGTGAATTTTTTGAGTTTCTTTTAAAATTTT	II
IFN-γ NF-kB	GGATCCCACTGGGTCTGGAACTCCCCCTGGGAATATTCTCT	II
IL-21 GAS	GGATCCGACATAGTTATTACCATAAGAAAAAGTCCT	III

5 RESULTS AND DISCUSSION

In this study the functions and regulatory role of a novel T cell-derived cytokine IL-21 on human peripheral blood derived T lymphocytes, NK cells and DC were studied. The experiments revealed the functions of IL-21 in the regulation of lymphocytes and in innate and adaptive immune responses.

5.1 Regulation of IL-21 and IL-21R expression in NK and T cells

5.1.1 IFN-α enhances TCR-mediated activation of IL-21 gene expression in T cells (III)

At present, TCR-activated T cells are the only known source of IL-21. Our results show that IFN-α/β increases TCR-induced IL-21 mRNA expression in human primary T cells. Naïve T cells are activated via TCR (CD3) during antigen presentation by APCs. TCR-mediated activation, however, is not sufficient to initiate T cell proliferation. In addition to TCR binding, the recognition of co-stimulatory molecules (B7-1, B7-2) by CD28 on T cells is also needed for full T cell activation. In vitro stimulation with anti-CD3 and anti-CD28 antibodies best mimics the antigen recognition of T cells and initiate their activation and proliferation process. It has been previously reported that T cell stimulation with anti-CD3 antibody induces IL-21 gene expression and combined stimulation with anti-CD3 and anti-CD28 even enhanced this expression. IL-21 expression is induced also by PMA+ionomycin stimulation but at lower level compared to TCR-mediated activation. No other stimulus, in addition to the ones mentioned above, has been reported to induce IL-21 mRNA expression. However, *in vivo* T cells, naïve and activated ones are constantly under regulation by several cytokines and other stimulatory molecules. Therefore, it is likely that at least some cytokines would regulate IL-21 production in T cells. Antigens are presented to naïve T cells in lymph nodes by APCs. The most potent antigen presenting cell in the lymph node is dendritic cell, which phagocytes microbes and matures before migrating to a local lymph node to present microbial antigens to naïve T cells. Infection with microbes, especially viruses, induces APCs to produce cytokines, among other type I interferon, IFN-α. Our studies revealed that IFN-α enhances the TCR-mediated upregulation of IL-21 gene expression. DC or other cell type-derived IFN-α thus increases IL-21 production from T cells, which have several stimulating effects on

NK and T cells. It is a novel finding that cytokines may contribute to the regulation of IL-21 production.

CD4+ T cells are producing IL-21 (159). The analysis of mouse Th cells, differentiated from lymph node or spleen-derived Thp cells, revealed that IL-21 is expressed in Th2 cells but not in Th1 cells (165). Mehta (160) and Kim (174) have analysed murine IL-21 promoter and by computer analysis they found putative binding sites for several transcription factors: three NFAT binding sites, several AP-1 consensus sites, a T-bet consensus site and a IRF-1 binding site. In their experiments the three NFAT binding sites turned out to be functional (regulating IL-21 transcription), although they had quite contradictory results about the specificity of these DNA binding elements in Th1/Th2 cells. In computer analysis we identified a putative STAT-binding site in the promoter region of IL-21 gene. Oligonucleotide DNA precipitation experiments revealed that this STAT binding site binded STATs after IFN-α stimulation. STAT binding to IL-21 promoter is likely to be one factor contributing to upregulation of IL-21 gene expression by IFN-α. However, the detailed analysis of the ability of this region to regulate IL-21 transcription requires further analysis.

5.1.2 IFN-α down-regulates IL-21R expression in NK and T cells (III)

As described above, IFN-α regulates IL-21 gene expression in T cells. It has been reported that IL-21 has multiple effects on NK and T cells, which express IL-21R on their surface. Interestingly we found out that IFN-α down-regulates the expression of IL-21R both in NK and T cells. NK-92 cells and T cells were stimulated with IFN-α or IL-12 and Northern blot analysis was performed. IFN-α inhibited IL-21R gene expression in both cell types. This reduces the responsiveness of these cells to IL-21 and thus limits the (activating) effects of IL-21 in these cells. The reduction in responsiveness to IL-21 was determined by analysing IL-21-induced STAT activation. Asao with coworkers (2001) and us (I) have reported that IL-21 mainly activates STAT3. Pre-treatment of NK cell line, NK-92 cells, with IFN-α inhibits IL-21-induced STAT3 phosphorylation and DNA binding as analysed with GAS element of CIS promoter. Thus IFN-α has a dual role in NK and T cell biology. On one hand it enhances IL-21 effects by increasing its production and on the other hand it limits IL-21-induced T cell activation by downregulating its IL-21R expression.

5.2 IL-21-induced gene expression in NK and T cells

Several reports describe IL-21 as a potent modulator of lymphocyte development and function (164, 166-169, 171, 175). Most of the studies have concentrated on the effects of IL-21 on development, differentiation, and maturation processes of NK and T cells. In this study the immunomodulatory effects of IL-21 were examined. We chose to analyze the expression of certain Th1 and innate immunity associated genes that are known to be important in immune responses against viral infections.

5.2.1 IL-21 increases the mRNA expression of Th1 type cytokine receptors (I, II)

IL-21 upregulates the expression of IL-2Rα, IL-12Rβ2, and IL-18R genes in NK and T cells. IL-2 is T cell-derived cytokine but recent studies have shown that it is produced also by dendritic cells in response to microbial activation and T-cell-mediated stimuli in the presence of IL-15 (172, 176, 177). IL-2R is comprised of three chains; IL-2Rα, IL-2Rβ,and γc and it thus belongs to the same receptor family with IL-21, sharing γc-receptor chain. IL-2 receptor chains can form heterodimeric complexes but IL-2Rα chain is an essential component to complete high-affinity functional IL-2 receptor (145). It has been reported that the expression of IL-2Rα chain is regulated by other γc-cytokines IL-2, IL-4, and IL-15 (143, 178, 179) and proinflammatory cytokines and IL-10 (180, 181). The best known action of IL-2 is to induce the proliferation of T cells in response to antigenic stimulation promoting both cytotoxic and suppressor T cells (182-184). In addition to its function as a T-cell growth and survival factor, IL-2 plays important role in induction of Fas-mediated activation of cell death of antigen restimulated T cells (185-187). IL-2Rα deficient mice are more susceptible to autoimmune disorders due to absent function of IL-2 (188). Accordingly, IL-2Rα expression regulates T cell growth and negatively regulates T cell-mediated immune responses. Our results show that IL-21 induces IL-2Rα expression in NK and T cells (I) and thus enhances IL-2 effects in these cells. The induction of IL-2Rα gene expression was fast, already after one hour stimulation IL-2Rα mRNA was detectable in NK-92 cells (I). In T cells IL-2Rα gene expression was induced at one hour time point but after three hours stimulation the expression reduced. IL-15, sharing receptor subunits with both IL-2 and IL-21, was used as a control since it is reported to induce IL-2Rα expression (178). Our results are in line with the finding that IL-15 induces IL-2Rα expression in T cells and also in NK cells (I).

IL-12 is a major Th1 type cytokine produced by antigen presenting cells (macrophages and DC) during microbial infection. The functional IL-12 receptor, which binds to IL-

12 with high affinity, is the heterodimeric receptor formend by IL-12Rβ1 and IL-12Rβ2 (189). IL-12Rβ1 is constitutively expressed on a large portion of resting T cells and is relatively unaffected by cytokines that induce Th1 or Th2 differentiation. On the other hand, the expression of IL-12Rβ2 is only detectable on activated T cells and is enhanced by cytokines that promote Th1 development and inhibited by cytokines that promote Th2 development (88, 112, 190). Therefore, regulating the expression of IL-12Rβ2 is a key step in modulating IL-12 function. In addition, IL-12Rβ2 plays important role in IL-12 signaling. Unlike IL-12Rβ1, the cytoplasmic domain of IL-12Rβ2 becomes tyrosine phosphorylated on IL-12 binding and provides binding sites for Tyk2 and Jak2. STAT1, STAT3, STAT4, and STAT5 are involved in IL-12 signaling in T cells but STAT4 is essential in mediating IL-12 function in T and NK cells (64, 191-193). IL-12 together with IFN-γ drives the adaptive immune response towards Th1 direction and antagonises the production and effects of Th2 cytokine IL-4. As described earlier, IL-12 and IL-18 co-operate to induce IFN-γ production in NK and T cells. We demonstrate that IL-21 stimulation of NK and T cells induces the expression of IL-12Rβ2 (I). This directs T cell immune response towards Th1 type and enhances innate immune responses mediated by NK cells. The increased responsiveness of NK and T cells to IL-12 also indirectly enhances IFN-γ production from NK and T cells. IL-21 thus promotes Th1 type response by enhancing IL-12 effects although IL-21 does not directly influence IL-12 production in DC (IV). IL-15 seems to differ from IL-21 in respect of ability to induce IL-12Rβ2 expression. IL-15 had no effect on inducing IL-12Rβ2 in NK cells but clearly induced IL-12Rβ2 expression in T cells (I).

IL-18R is comprised of two receptor chains, IL-18Rα and IL-18Rβ (123, 124). IL-18Rα belongs to the IL-1R family. These receptors use IL-1R-associated kinase (IRAK) – NF-kB signalling pathway. The activation and signal transduction of this pathway requires an adapter protein MyD88 (125, 194). Our results show that IL-21 (and IL-15) enhances the expression of both IL-18R chains and MyD88 adaptor molecule (I). IL-18 in synergy with IL-12 enhances Th1 type cytokine, IFN-γ, production in NK and T cells. IL-21 thus promotes Th1 immune responses.

5.2.2 IL-21 activates Jak/STAT pathway (I, II)

In the first report in the literature describing IL-21R, the ligand was still unknown (162). So the authors created a chimeric receptor construct containing the extracellular domain of the erythropoietin receptor (EPOR) and the transmembrane and cytoplasmic domains of IL-21R, or a novel interleukin receptor (NILR), as they named it. They observed that EPOR/NILR chimera mediated the proliferation of Ba/F3 (B cell line) cells in the presence of EPO. They also analyzed the STAT

proteins that were activated by EPOR/NILR homodimerization. The result was that EPOR/NILR activates Jak1 and STAT5a and STAT5b but no other STATs. However, further studies by Asao and coworkers (141) showed that in HTLV-I transformed human T cell line γc-receptor chain is necessary subunit of IL-21R and that IL-21R signalling involves Jak1/Jak3 tyrosine phosphorylation and activation of STAT1, STAT3 but not STAT5. These observations were inconsistent with those of Ozaki et al. After receiving the above mentioned data conserning IL-21-induced gene expression and these diverse results about IL-21R signalling we carried out experiments with human primary T cells and NK-92 cells to clarify this question. Our studies revealed that stimulation with IL-21 activates tyrosine phosphorylation of Jak1 and Jak3 but not Jak2 or Tyk2 (I). This was consistent with previous studies by Ozaki and Asao. In addition, IL-21 stimulation of NK and T cells induced the activation of STAT1, STAT3, and STAT4 but not that of STAT5. STAT activation was studied further by analysing STATs binding to the promoter regions of the genes it upregulates, such as IL-2Rα, MyD88, and IFN-γ. IL-21-induced STAT3 binding was observed to the promoter of MyD88 GAS and SIE elements (I). IL-15 induced STAT5 binding to IL-2Rα promoter GAS site in NK-92 cells and this was also confirmed by supershift analysis (I).

IL-21 also induces STAT3 DNA binding to the GAS elements of IFN-γ promoter and its 1st intron. However, IL-21 does not activate NF-kB binding to IFN-γ promoter whereas IL-18 is able to induce NF-kB binding to IFN-γ promoter NF-kB site. In addition to STAT3, IL-21 is able to activate at least to some extent STAT1 and STAT4 tyrosine phosphorylation and binding to IFN-γ promoter GAS. Furthermore, pre-treatment of NK and T cells with IL-21 enhances IL-12-induced STAT4 activation. This is either because of the direct activation of STAT4 by IL-21 or because IL-21 increases the expression of IL-12R on the surface of NK and T cells, and therefore IL-21 pre-treated cells are more responsive to IL-12.

5.2.3 IL-21-induced IFN-γ expression (I,II)

In addition to IL-12, IFN-γ is the most important Th1 type immunity promoting cytokine. It drives Th differentiation towards Th1 direction and antagonises IL-4 effects, reviewed in (105). IL-21 stimulation was able to induce IFN-γ mRNA expression in NK and T cells (I). Induction of IFN-γ mRNA expression was rapid. It could be seen after one hour stimulation. In NK-92 cells IFN-γ gene expression lasted up to nine hours but in T cells it started to diminish already at three hours after stimulation. This suggests that IFN-γ gene expression is tightly regulated in T cells, although the exact mechanisms were not examined in this study. T-bet is a transcription factor that has been shown to regulate IFN-γ production in NK and T

cells (195-197). T-bet and IFN-γ form a positive feed-back loop in which they enhance each others gene expression (197-199). IL-21 enhanced T-bet gene expression in NK and T cells (I). Enhanced T-bet gene expression was detectable already after one hour IL-21 stimulation, which refers to direct induction by IL-21 rather than IFN-γ-mediated activation. In these experiments the later time points were not analysed but it is likely that later, when IFN-γ and T-bet proteins are produced, they would positively regulate the gene expression of each other. IFN-γ gene expression was induced also by IL-15 (I). In NK-92 cells the expression peaked at the three hour time point and then rapidly diminished. In T cells IL-15 induced IFN-γ gene expression already one hour after stimulation. Similar to NK-92 cells in T cells IFN-γ mRNA expression was reduced three hours after stimulation.

NK-92 and T cells were also stimulated with a combination of IL-21 and IL-15 or IL-18 and IFN-γ production was analyzed (II). Cell culture supernatants from cells stimulated with either cytokine (IL-21, IL-15, IL-18) alone or their combination were collected and ELISA tests were performed. In primary NK cells IL-15 was the only cytokine that was able to induce detectable IFN-γ production. In NK-92 cells and T cells each cytokine individually induced IFN-γ production, IL-15 being again the strongest inducer. When IL-15 was combined with either IL-18 or IL-21, IFN-γ production was markedly increased. Combination of IL-15 and IL-18 was in every cell type more effective compared to IL-15 plus IL-21. IL-21 combined with IL-18 also significantly increased IFN-γ production compared to these cytokines separately. The same effect was detectable in mRNA level when mRNA from the above described experiments was isolated and Northern blot analysis was carried out (II). The promoter of IFN-γ gene contains several transcription factor binding sites including AP-1, NF-κB, and NFAT. These factors regulate IFN-γ gene expression in response to TCR and/or cytokine stimulus. IFN-γ gene also contains putative STAT binding sites (GAS) in the promoter and in the first and third introns. Oligonucleotide immunoprecipitation assay was used to analyse the STAT binding to these regulatory elements of IFN-γ gene. IL-21 and IL-15 induced STAT DNA binding to IFN-γ promoter GAS and 1st intron GAS in NK-92 and primary NK cells (II). IL-21 induced binding of STAT1, STAT3, and STAT4 but not that of STAT5. IL-15 induced binding of STAT1, STAT3, and especially STAT4 and STAT5 to both elements. In primary NK cells IL-21 activated STAT1 and STAT3 and IL-15 activated mostly STAT5 and these factors bound to the IFN-γ promoter GAS. Similar results were obtained from experiments carried out in T cells. IL-18 on the other hand did not induce any STAT binding to these regulatory elements of IFN-γ gene. Instead, IL-18 induced NF-kB binding to IFN-γ promoter NF-κB site (II). In the oligonucleotide immunoprecipitation experiments p50 and p65 proteins were found to bind to IFN-γ NF-κB site rapidly after IL-18 stimulation. IL-15 induced NF-kB binding with a slower kinetics. The binding of NF-kB was detectable nine

hours after IL-15 stimulus. IL-21, for one, did not induce NF-kB binding to IFN-γ promoter NF-kB site. IL-21, IL-15, and IL-18 induce thus differential binding of STATs and NF-kB to IFN-γ gene regulatory elements. This provides a possible explanation for the synergistic effects of these cytokines in IFN-γ gene expression. IL-21 and IL-15 activate different STATs that bind to STAT binding regions in the promoter and introns of IFN-γ gene. IL-18 activates binding of NF-kB, which binds to NF-kB site in the promoter of IFN-γ gene. This concurrent activation and binding of several transcription factors to IFN-γ gene, results in enhanced transcription and production of IFN-γ. IL-15 and IL-18 are APC-derived cytokines, which are produced in the early phases of immune response. These cytokines individually and especially in combination enhance IFN-γ production from NK and T cells. The combination of these three cytokines further enhance IFN-γ gene expression and thus promote innate immune responses by activating phagocytosis and promote Th1 response by activating antigen presentation by APC .

5.3 IL-21 modulates DC functions

5.3.1 IL-21R is differentially expressed in human monocyte derived macrophages and DC (IV)

Macrophages and dendritic cells can be differentiated from monocytes in the presence of GM-CSF or GM-CSF plus IL-4, respectively. To current knowledge, it is not known whether IL-21R is expressed in human monocyte derived macrophages and dendritic cells. Report by Brandt et al.
murine bone marrow derived DC and thus it was likely that it would be expressed in human DCs as well. Monocytes were isolated from buffy coat preparations and differentiated into macrophages or DCs. Total cellular RNA was isolated from 1d old monocytes and cells differentiated for 3 or 7 days. Macrophages failed to express IL-21R mRNA at any time point of their differentiation. In DCs instead, IL-21R mRNA expression was induced already at day 3 and it increased by day 7 (IV). The mRNA expression of γc, the other subunit of IL-21 receptor complex, was high in 1d old monocytes but it decreased during differentiation both in macrophages and DC.

5.3.2 DC maturation and cytokine production is inhibited by IL-21 (IV)

Immature DCs reside in pheripheral tissues and are on alert for invading microbes or antigens. After recognising the microbe, the DC maturation process starts. The maturation process is associated with increased expression of co-stimulatory molecules, decreased antigen intake, and enhanced secretion of cytokines and

chemokines. In the mouse model, DCs are blocked in an immature stage if the cells are exposed to IL-21 during the differentiation with GM-CSF (200). IL-21-differentiated cells exhibit reduced levels of CD80, CD86, and HLA-II molecules on their surface, increased antigen intake, and reduced cytokine production levels as compared to IL-21-unexposed cells. In addition, IL-21-differentiated DCs fail to activate naïve T cells (201). Our results show that human monocyte-derived DCs have similarities but also differences with their murine counterparts. Human DCs are differentiated *in vitro* in the presence of GM-CSF+IL-4 whereas murine DCs differentiate with GM-CSF. When human monocytes are treated with GM-CSF only, macrophages are obtained. Human monocytes treated with GM-CSF+IL-21 differentiated neither to DCs nor macrophages. Thus the *in vitro* differentiation of monocytes to DCs requires the presence of both GM-CSF and IL-4 and the possible inhibitory effects of IL-21 on human DC differentiation can not be analyzed.

IL-21-pretreatment inhibited LPS-induced maturation in GM-CSF+IL-4-differentiated immature DCs. Maturation of DCs was detected by analyzing the expression of co-stimulatory molecules on the surface of DCs. CD86 expression was markedly reduced in IL-21-pretreated cells. HLA-II expression was also inhibited but the difference was not statistically significant compared to untreated controls. LPS-induced cytokine and chemokine production was also diminished by IL-21 pre-treatment. TNF-α, IL-12, CCL5, and CXCL10 secretion occurred at lower level in IL-21-DCs compared to untreated cells. Reduction in the expression of IL-12, a crucial Th1 type cytokine is likely to reduce Th1 polarization of T cells. In addition, CXCL10 is a crucial chemoattractant of Th1 type cells and thus IL-21 confines Th1 response also in this way.

5.3.3 IL-21 induces SOCS gene expression (IV)

LPS-induced activation of DC is mediated through TLR4 signaling. Binding of LPS to TLR4 leads to activation of signalling cascades, which in the end lead to binding of IRFs and NF-κB to specific response elements in their target genes. IL-21 pre-treatment did not have any markable effect on the expression of TLR4 or its downstream signalling components (IV). SOCS proteins are suppressing cytokine signalling but they have also been reported to negatively regulate TLR signalling. Several groups have reported that SOCS-1 inhibit TLR4-mediated signalling (202-204). SOCS-1 has been shown to negatively regulate DC maturation. SOCS-$1^{-/-}$ DCs exhibited a more mature phenotype, enhanced antigen presentation to Th1 cells, were hyperresponsive to LPS, and induced autoreactive antibody production (205-208). As described above, IL-21 did not reduce the expression of TLR4 or its downstream signalling components but instead enhanced the expression of SOCS

genes. DCs were stimulated with IL-21 or IFN-γ and Norther blot analysis was performed. IL-21 induced SOCS-1 and SOCS-3 expression in DCs (IV). As IFN-γ is known inducer of SOCS gene expression (reviewed in (78) we compared IL-21-induced SOCS expression to that of IFN-γ. Congruent with previous reports, IFN-γ induced SOCS-1 and SOCS-3 gene expression. IL-21 and IFN-γ appeared to induce SOCS-1 gene equally well, whereas IFN-γ was somewhat better inducer of SOCS3 as compared to IL-21. Cytokine stimulations did not regulate SOCS-2 and CIS mRNA levels. The observed induction of SOCS-1 (and SOCS-3) by IL-21 is a novel finding that provides one possible explanation for perceived inhibition of DC maturation and LPS-induced responses.

6 CONCLUSIONS

Cytokines are molecules that mediate signals between cells of the immune system and they have multiple effects on their target cells. Each cytokine may have several target cells but its effect in each cell type may differ. In addition, the same cytokine may have different effects on the same cell type depending on the cytokine milieu or other stimuli that may occur around the cell. Cytokine ligand binding to its specific receptor leads to activation of signal transduction cascades, which finally results in activation or repression of target gene expression.

IL-21 was characterized in year 2000 and since then great amount of information has been gathered about its function and regulatory role in immune system. The principal aim of the present study was to characterize the regulatory functions of IL-21 in human leukocytes. Firstly, IL-21 and IL-21R expression was analyzed in NK and T cells. Our results show that IFN-α enhances IL-21 production in T cells. Interestingly, IFN-α also seems to negatively regulate IL-21 effects by down-regulating IL-21R in NK and T cells. The second aim was to analyze IL-21-induced gene expression in NK and T cells. IL-21 enhances the expression of cytokine receptors that are associated with innate immunity and Th 1 type immune responses. IL-21 also enhanced, synergistically with IL-15 or IL-18, IFN-γ production from NK and T cells, which is known to effectively drive T cells towards Th1direction. The next aim of the study was to clarify the transcription factors that are activated during IL-21 stimulation. We concentrated on Jak-STAT pathway because that is the most common pathway activated after cytokine stimulus. IL-21 activated Jak1 and Jak3 and further STAT1, STAT3, and STAT4. In the fourth study we analyzed the effects of IL-21 on DC maturation and activation. IL-21 blocks DC in immature stage. LPS response in IL-21-pre-treated DCs is inhibited but it is not due to reduced expression of TLR4 or its downstream signalling molecules. Instead, IL-21 induces SOCS1 and SOCS3 expression in DC. SOCS proteins are known to downregulate both TLR signalling and DC maturation.

The results provide important information of IL-21 functions in human leukocytes. It, at least in human system, promotes innate and Th1 type immune responses by upregulating Th1 associated genes in NK and T cells. However, IL-21 inhibits DC maturation and cytokine production, which reduces DC's capability to present antigens to naïve T cells.

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