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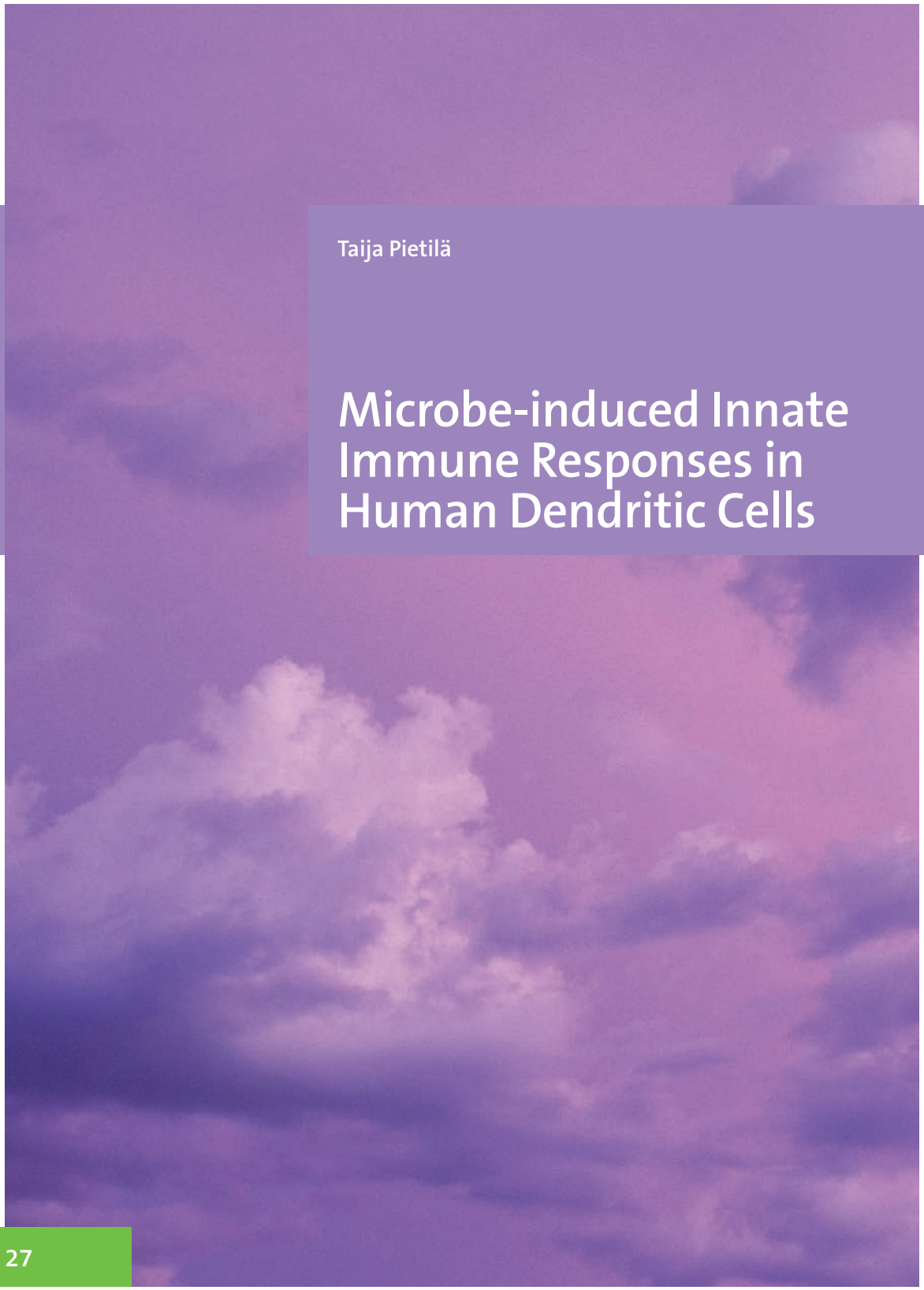

RESEARCH

Taija Pietilä

Microbe-induced Innate Immune Responses in Human Dendritic Cells

RESEARCH 27

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ACADEMIC DISSERTATION

To be presented with the permission of the Faculty of Biological and Environmental Sciences, for public examination in the Arppeanum auditorium, Helsinki University Museum, Snellmaninkatu 3, Helsinki, on January 22nd, at 12 o'clock noon.

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Helsinki 2009

ISBN 978-952-245-128-6 (printed)
ISBN 978-952-245-208-5 (pdf)
ISSN 1798-0054 (printed)
ISSN 1798-0062 (pdf

Helsinki University Print
Helsinki, Finland 2009

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Abstract

Taija Pietilä. Microbe-induced Innate Immune Responses in Human Dendritic Cells. National Institute for Health and Welfare (THL), Research 27/2009. 154 pages. Helsinki 2009. ISBN 978-952-245-128-6 (printed), ISBN 978-952-245-208-5 (pdf)

Two types of antigen-presenting cells (APCs), macrophages and dendritic cells (DCs), function at the interface of innate and adaptive immunity. Through recognition of conserved microbial patterns, they are able to detect the invading pathogens. This leads to activation of signal transduction pathways that in turn induce gene expression of various molecules required for immune responses and eventually pathogen clearance. Cytokines are among the genes induced upon detection of microbes. They play an important role in regulating host immune responses during microbial infection. Chemotactic cytokines, chemokines, are involved in migratory events of immune cells. Cytokines also promote the differentiation of distinct T cell responses. Because of the multiple roles of cytokines in the immune system, the cytokine network needs to be tightly regulated.

In this work, the induction of innate immune responses was studied using human primary macrophages or DCs as cell models. *Salmonella enterica* serovar Typhimurium served as a model for an intracellular bacterium, whereas Sendai virus was used in virus experiments. The starting point of this study was that DCs of mouse origin had recently been characterized as host cells for *Salmonella.* However, only little was known about the immune responses initiated in *Salmonella*-infected human DCs. Thus, cellular responses of macrophages and DCs, in particular the pattern of cytokine production, to *Salmonella* infection were compared.

Salmonella-induced macrophages and DCs were found to produce multiple cytokines including interferon (IFN) -γ, which is conventionally produced by T and natural killer (NK) cells. Both macrophages and DCs also promoted the intracellular survival of the bacterium. Phenotypic maturation of DCs as characterized by up-regulation of costimulatory and human leukocyte antigen (HLA) molecules, and production of CCL19 chemokine, were also detected upon infection with *Salmonella*.

Another focus of this PhD work was to unravel the regulatory events controlling the expression of cytokine genes encoding for CCL19 and type III IFNs, which are central to DC biology. We found that the promoters of CCL19 and type III IFNs contain similar regulatory elements that bind nuclear factor κB (NF-κB) and interferon regulatory factors (IRFs), which could mediate transcriptional activation of the genes. The regulation of type III IFNs in virus infection resembled that of type

I IFNs – a cytokine class traditionally regarded as antiviral. The induction of type I and type III IFNs was also observed in response to bacterial infection.

Taken together, this work identifies new details about the interaction of *Salmonella* with its phagocytic host cells of human origin. In addition, studies provide information on the regulatory events controlling the expression of CCL19 and the most recently identified IFN family genes, type III IFN genes.

Keywords: Cytokines, dendritic cell, innate immunity, *Salmonella*, transcription factor

Abstract in Finnish

Taija Pietilä. Microbe-induced Innate Immune Responses in Human Dendritic Cells [Mikrobien aikaansaama synnynnäisen immuniteetin aktivoituminen ihmisen dendriittisoluissa]. National Institute for Health and Welfare (THL), Research 27/2009. 154 pages. Helsinki 2009. ISBN 978-952-245-128-6 (printed),
ISBN 978-952-245-208-5 (pdf)

Kehomme immuunijärjestelmä on jakautunut synnynnäiseen ja hankittuun immuniteettiin, jotka yhdessä vastaavat mikrobi-infektioiden torjunnasta. Luonnollinen ja hankittu immuniteetti ovat vuorovaikutuksessa toistensa kanssa antigeeniä esittelevien solujen kuten makrofagien ja dendriittisolujen välityksellä. Makrofagit ja dendriittisolut tunnistavat evoluutiossa säilyneitä mikrobirakenteita, jotka toimivat varoitussignaalina puolustusjärjestelmien aktivoitumiselle. Eräs tärkeä ryhmä proteiineja, joiden tuotanto käynnistyy solunsisäisten signaalinvälitysreittien toimesta, ovat sytokiinit. Monet sytokiineistä ohjaavat T-soluvasteiden erilaistumista, kemokiinien päätehtävänä on puolestaan säädellä valkosolujen liikennettä kudoksissa. Lukuisten tehtäviensä vuoksi on tärkeää, että sytokiinijärjestelmä on tiukan säätelyn alainen.

Tässä työssä tutkittiin synnynnäisen immuniteetin aktivoitumista ihmisen makrofageissa ja dendriittisoluissa. Mikrobi-infektion mallintamisessa käytettiin *Salmonella enterica* sevorar Typhimurium -bakteeria sekä Sendai-virusta. Työ aloitettiin, koska haluttiin selvittää toimivatko ihmisen dendriittisolut *Salmonella*-bakteerin isäntäsoluna, ja miten erityisesti sytokiinituotanto aktivoituu infektion aikana. Vertailukohtana käytettiin makrofageja, joiden toiminnasta *Salmonella*-infektiossa tiedettiin jo paljon ennen tätä tutkimusta.

Salmonella sai aikaan makrofageissa ja dendriittisoluissa useiden sytokiinien tuotannon mukaan lukien interferoni (IFN)-γ:n, jota yleensä tuottavat luonnolliset tappajasolut ja T solut. *Salmonella* pystyi selviytymään paitsi makrofagien myös dendriittisolujen sisällä. Lisäksi CCL19-kemokiinin erittyminen ja tiettyjen antigeeniesittelyssä tarvittavien molekyylien ilmentyminen solun pinnalla kuvastivat dendriittisolujen kypsymistä *Salmonella*-infektion aikana.

Tutkimuksessa tarkasteltiin myös tarkemmin miten sytokiinigeenien ilmentyminen on säädelty transkriptiotasolla. CCL19- ja tyypin III IFN -geenien promoottorialueilta löydettiin samankaltaisia säätelyelementtejä, joiden havaittiin sitovan NF-κB- ja IRF-transkriptiotekijöitä välittäen myös geenien aktivaatiota. Osoitimme myös, että tyypin I ja tyypin III IFN -geenien säätely muistuttavat

toisiaan virusinfektiossa. Tyypin I ja III IFN -tuotantoa on aiemmin tutkittu pääasiallisesti virusinfektion aikana, mutta tuloksemme osoittavat, että niitä ilmennetään myös bakteeri-infektion seurauksena.

Tämä väitöskirjatyö tuo esille uutta tietoa *Salmonella*-bakteerin ja sen isäntäsolujen, makrofagien ja dendriittisolujen, välisestä vuorovaikutuksesta. Lisäksi tutkimukset syventävät tietämystämme miten dendriittisolujen biologiassa tärkeiden CCL19- ja tyypin III IFN geenien aktivaatiota säädellään mikrobi-infektion aikana.

Avainsanat: dendriittisolu,synnynnäinen immuniteetti, *Salmonella*, sytokiini, transkriptiotekijä

List of original papers

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

I Pietilä TE, Veckman V, Kyllönen P, Lähteenmäki K, Korhonen TK, Julkunen I. 2005. Activation, cytokine production, and intracellular survival of bacteria in *Salmonella*-infected human monocyte-derived macrophages and dendritic cells. *J Leukoc Biol.* 78:909-20.

II Pietilä TE, Veckman V, Lehtonen A, Lin R, Hiscott J, Julkunen I. 2007. Multiple NF-kappaB and IFN regulatory factor family transcription factors regulate CCL19 gene expression in human monocyte-derived dendritic cells. *J Immunol.* 178:253-61.

III Pietilä TE, Latvala S, Österlund P, Julkunen I. Inhibition of dynamin-dependent endocytosis interferes with type III IFN expression in bacteria-infected human dendritic cells. Submitted for publication.

IV Österlund PI, Pietilä TE, Veckman V, Kotenko SV, Julkunen I. 2007. IFN regulatory factor family members differentially regulate the expression of type III IFN (IFN-lambda) genes. *J Immunol.* 179:3434-3442.

Abbreviations

AP-1	activator protein 1
APC	antigen-presenting cell
ATCC	American Type Culture Collection
CARD	caspase recruitment domain
CARDIF	CARD adaptor inducing IFN-β
CD	cluster of differentiation
CHX	cycloheximide
DC	dendritic cell
ds	double-stranded
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
GM-CSF	granulocyte-macrophage colony stimulating factor
HEK	human embryonic kidney
HLA	human leukocyte antigen
IFN	interferon
IKK	inhibitory κB kinase
IL	interleukin
IRF	interferon regulatory factor
ISG	IFN-stimulated gene
ISGF3	interferon-stimulated gene factor 3
ISRE	interferon-stimulated response element
JAK	Janus kinase
LPS	lipopolysaccharide
LTA	lipoteichoic acid
MAPK	mitogen-activated protein kinase
mDC	myeloid dendritic cell
MHC	major histocompatibility class
mo-DC	monocyte-derived dendritic cell
MOI	multiplicity of infection
MyD88	myeloid differentiation primary-response gene 88
NF-κB	nuclear factor kappa-B
NK	natural killer
NLR	nucleotide-binding domain and leucine-rich repeat containing gene family
NLRP3	NLR family, pyrin domain containing 3
NOD	nucleotide-binding oligomerization domain
PAMP	pathogen-associated molecular pattern

PBMC	peripheral blood mononuclear cell
pDC	plasmacytoid dendritic cell
PG	peptidoglycan
PRD	positive regulatory domain
PRR	pattern recognition receptor
RIG-I	retinoic acid-inducible gene I
RLR	RIG-I-like receptor
SDS	sodium dodecyl sulphate
SPI	*Salmonella* pathogenicity island
ss	single-stranded
STAT	signal transducer and activator of transcription
TBK1	Tank-binding kinase1
Th	T helper
TIR	Toll/IL-1 receptor
TLR	Toll-like receptor
TNF	tumor necrosis factor
TRIF	TIR domain-containing adaptor protein inducing IFN-β

Contents

1 Introduction

Mammalian hosts are constantly in danger for the invasion of numerous pathogenic microbes. Although the host's internal milieu is well protected by barriers such as skin and mucosal tissues, pathogens have evolved several strategies to circumvent such obstacles. Therefore, microbial infections must be limited by rigorous host defense mechanisms.

The initial encounter between the host and the microbe rapidly activates innate immunity. The key players of this arm of immunity are complement, natural killer (NK) cells that destroy infected cells, as well as neutrophils, macrophages and dendritic cells (DCs), which actively take up microbes. The relatively non-specific activation of innate immunity depends on evolutionarily conserved pattern recognition receptors (PRRs) that are able to discriminate self from non-self structures.

The omnipresent innate mechanisms are accompanied by activation of adaptive immunity, which provides efficient cytotoxic responses against the microbes as well as immunological memory and protective immunity. Unlike the innate responses, adaptive immunity is highly specific due to a vast repertoire of clonally selected receptors, and is activated in the late phase of infection. The generation of adaptive immune responses relies on the functions of T and B cells.

The communication between the different types of immunity is primarily mediated by antigen presenting cells (APCs) including macrophages and DCs. In particular, DCs possess the capacity to prime naïve T cells. In response to infection, APCs also produce cytokines that, besides exerting direct antimicrobial functions, act as key messengers between immune effector cells. Cytokines regulate immune responses in multiple ways, thus their production needs to be firmly controlled when necessary.

2 Review of the literature

2.1 Phagocytic, antigen-presenting cells of the immune system

Phagocytosis, the uptake process of microbes and other large particles, represents one of the major mechanisms our innate immune system utilizes for fighting against pathogenic microbes. Professional phagocytes such as neutrophils, macrophages and DCs express phagocytic receptors for internalization of foreign particles including pathogens and apoptotic cells. However, only macrophages and DCs can efficiently act as APCs, and thereby contribute to the initiation of adaptive immune responses.

2.1.1 Macrophages

Macrophages are found in virtually all tissues. They differentiate from monocytes circulating in the peripheral blood. This monocyte cell population originates from a common myeloid progenitor cell in the bone marrow that is the precursor of many other cell types including neutrophils and DCs. Monocytes leave the blood circulation and enter different tissues where they display remarkable heterogeneity as macrophages in both functional and phenotypic aspects (1).

Macrophages are vital both in maintaining tissue homeostasis through the clearance of apoptotic cells and tissue remodeling as well as activation of immune responses during microbial infections. The characteristic functional activities and set of surface receptors of a given macrophage population reflects the adaptation to different anatomical locations (1, 2). Kupffer cells in the liver, microglia of the central nervous system, and osteoclasts with bone resorption activity are all representatives of highly differentiated and specialized macrophage populations.

Upon microbial infections, macrophages detect and internalize pathogens, and initiate T cell responses by antigen presentation (3). Macrophage receptors involved in immune recognition include cluster of differentiation (CD) 14, complement, scavenger and Fc-receptors, PRRs such as Toll-like receptors (TLRs), and lectins (4). Many of these receptors also mediate phagocytosis and trigger the activation of complex signaling cascades (5).

Macrophages act primarily to destroy pathogens and are highly important in host defense to intracellular microbes. Their microbicidal activity is enhanced by cytokines, particularly interferon (IFN)-γ, produced by natural killer (NK) and T

cells. Consequently, macrophages themselves produce high levels of pro-inflammatory mediators that influence the following immune response (1, 6).

Intriguingly, some bacteria have evolved to survive the harsh intracellular milieu of macrophages. *Listeria*, *Legionella*, and *Salmonella* are good examples of such bacteria (5, 7).

2.1.2 Dendritic cells

DCs reside in peripheral tissues and lymphoid organs and sense the surrounding environment for invading microbes. This specialized cell has diverse origins; both myeloid and lymphoid progenitor cell lines can create different DC subtypes. In humans, myeloid DCs (mDCs) are found as Langerhans cells and interstitial DCs in the epidermal and dermal layers of the skin, respectively. Also secondary lymphoid organs and circulating blood contain subsets of mDCs. Plasmacytoid DCs (pDCs) of lymphoid origin travel in blood circulation (8). In mice, at least six different subtypes of DCs exist, although the precise definition of a subtype is still unclear (9). Conventional DCs can be subdivided based on the expression of lymphoid and myeloid lineage surface molecules or microenvironmental localization. pDCs and tumor necrosis factor (TNF) -α/nitric oxide producing DCs are representatives of non-conventional mouse DCs (9, 10).

The research of DC biology was for years hampered by the unavailability of *in vitro* cultivation techniques. The discovery that human monocytes could be differentiated into so-called monocyte-derived DCs (mo-DCs) in the presence of granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin (IL)-4 (11), has led to a rapid accumulation of data on molecular and biological characteristics of DCs. The *in vitro* culture of human monocytes in the presence of GM-CSF alone yields monocyte-derived macrophages. Gene expression profiling has proven monocyte-derived macrophages and DCs to be quite different from one another (12). Although useful for studying DC biology, it remains unclear whether mo-DCs have counterparts *in vivo* (13).

For detection of microbial components, DCs are armed with a wide repertoire of PRRs, the expression of which varies between different DC subtypes (8). Similar to macrophages, the cell surface of DCs is decorated with phagocytic receptors coupled to the internalization of pathogens (14, 15).

Unique to DCs is that in order to be fully efficient APCs and thus capable of educating T cells, they must undergo a controlled maturation program. This is initiated upon DC contact with a microbe or inflammatory message from

environment. The process in general is characterized by morphological changes in cellular structure, down-modulation of endocytosis/phagocytosis, upregulation of costimulatory and MHC (human leukocyte antigen; HLA in humans) molecules, and secretion of cytokines (8, 14).

DC maturation is often accompanied by migration of the cells from the periphery into secondary lymphoid tissues where antigen presentation takes place. DCs present endogenous and exogenous antigens in the context of MHC I and MHC II molecules, respectively (15). In addition, DCs are known to present exogenous antigens also by MCH I molecules, which is referred to as cross-presentation. Since DCs are the most efficient APCs, and they exclusively possess the ability to prime naïve T cells (both $CD4^+$ and $CD8^+$), they play a major role in initiating adaptive immune responses. In addition to inducing antigen-specific T cell responses, DCs can activate NK cells, natural killer T cells, and humoral responses in B cells (14, 16).

The interaction of DCs and T cells involves a complex hierarchy of molecules. MHC molecules are first recognized by T cell receptor on antigen-specific T cells. This 'signal one' is not sufficient to activate immune responses. 'Signal two' constitutes the interaction of costimulatory molecules such as CD80 and CD86 on DCs with CD28 on T cells. 'Signals one and two' together promote immune responses, whereas signal one alone has been suggested to induce tolerance. 'Signal three' refers to the APC-derived mediators such as cytokines that direct the development of T cell responses into different types such as T helper (Th) 1 or Th2 (17).

Cytokines exhibit dual functions in the interaction of DC and T cells. On the one hand, pro-inflammatory cytokines and IFNs produced by innate immune cells modulate DC function and, on the other hand, cytokines secreted by DCs promote T cell differentiation. IL-12 produced by DCs favors Th1 type response important against intracellular pathogens (18, 19). Th17 cell-mediated immunity against extracellular bacteria and fungi require IL-23, another IL-12 family member produced by DCs. Th2-type responses essential against parasitic infections, and the induction regulatory T cells critical in maintaining tolerance, can also be promoted by DCs (19-21).

Viruses, bacteria, and parasites have evolved several strategies to impede DC functions in host defense (8, 22). Many microbes actively block DC maturation, whereas some viruses induce apoptotic cell death in DCs. Bacterial toxins delivered into the phagocyte can destroy DCs. Some pathogens exploit host cell receptors for their entry and thereby spread in the host. In contrast, some microbes inhibit the migration of DCs into the proximity of effector immune cells. A clever way to

exploit DCs is also the ability of microbes to switch from the protective cytokine responses to aberrant, non-protective type.

Since DCs are central to multiple immune functions, the dysregulation of this finely controlled cell system poses several threats for human beings. DCs are implicated in autoimmune diseases such as systemic lupus erythematosus, multiple sclerosis and allergies. However, DCs can also be targeted in therapy and used as tools for vaccination (22).

2.2 Innate pathogen recognition

Activation of innate immune responses depends on rapid recognition of invading pathogens. Thus, innate immune cells including phagocytic macrophages and DCs are equipped with multiple receptor families that detect conserved microbial patterns, so called pathogen-associated molecular patterns (PAMPs) (Table 1). The PRRs detecting PAMPs can be divided into three major classes: TLRs, nucleotide-binding and leucine-rich repeat containing gene family receptors (NLRs), and retinoic acid-inducible gene I (RIG-I) like receptors (RLRs) (Fig. 1.). TLRs, NLRs, and RLRs are not thought to directly mediate phagocytosis; in contrast, they determine the type of microbe and class of infection to control activation of adaptive immune responses. For this purpose, they activate intracellular signaling cascades resulting in the expression of many genes important in inflammatory and immune responses.

2.2.1 Toll-like receptors

Mammalian immune system comprises at least 12 TLR members, out of which TLRs 1-10 have been identified in humans (23, 24). The importance of the TLR system in innate immunity was originally discovered in *Drosophila*, when Lemaitre and co-workers showed that the Toll protein played a major role in antifungal defenses (25). Afterwards, the specific pathogen ligands for most TLRs have been found (Table 1.), and for some interacting receptor-ligand pairs even crystal structures have been determined (23).

TLR molecules are type I transmembrane proteins that contain leucine-rich repeat and Toll/IL-1 receptor (TIR) domains for recognition of PAMPs and initiation of intracellular signaling, respectively. TLRs can be classified according to their cellular location. TLRs 1, 2, 4, 5, 6, and 11 are expressed on the cell surface, whereas TLRs 3, 7, 8, and 9 are found in intracellular locations such as endosomes or lysosomes (23, 24). TLR expression in general is ubiquitous among immune cells,

and can be modulated in response to pathogens, cytokines, and stress factors. However, the distinct set of TLRs in blood mDCs and pDCs is an example reflecting the specialization of these cell types to respond to certain stimuli (26, 27).

Bacteria are recognized by a wide repertoire of TLRs. Defective responses to lipopolysaccharide (LPS) in mice strains carrying mutations in the *Tlr4* gene led to the discovery of TLR4 (28). LPS is the major component of the cell wall in Gram-negative bacteria and responsible for the septic shock. TLR2 is involved in the detection of peptidoglycan (PG), lipoproteins and lipoteichoic acid, and is considered more important in immune responses against Gram-positive bacteria. TLR2 can interact with TLR1, TLR6, and non-TLR molecules to discriminate the molecular properties of the ligands (23, 29, 30). Extracellular flagellin, a structural component of bacterial motility apparatus, is detected by TLR5 (31-33). TLR11 has been implicated in the recognition of uropathogenic bacteria (34), but the human ortholog is nonfunctional.

The genomic material of bacteria is recognized via TLR7 and TLR9. The presence of unmethylated CpG motifs in prokaryotic DNA was originally described as the basis for TLR9 operation (35). Later it has been shown that the phosphodiester 2' deoxyribose backbone of single-stranded (ss) DNA is sufficient to confer TLR9 activation (36) and that the discrimination between self and non-self DNA is feasible due to the endosomal localization of TLR9 (37). Recent work by Mancuso et al. (38) has demonstrated that bacterial RNA can be sensed by TLR7 in conventional DCs. Similar role for TLR7 in recognition of bacterial RNA has been suggested in pDCs (39). Thus, all known TLRs except TLRs 3, 8, and 10 have reported functions in the recognition of bacterial ligands.

The genomic material of viruses also serves as the major target for TLR family members. TLR3 detects double-stranded (ds) RNA (40), TLRs 7 and 8 sense ssRNA (41, 42), and TLR9 recognizes the genetic material of some DNA viruses (43). In addition, TLRs 2 and 4 have been implicated in detection of proteins and unknown ligands of viral origin (23).

TLR signaling is mediated via multiple adaptor molecules including myeloid differentiation primary response gene 88 (MyD88), TIR domain-containing adaptor, TIR domain-containing adaptor protein inducing IFN-β (TRIF), and TRIF-related adaptor molecule (Fig. 1.). After ligand binding, the TIR domains of TLRs and adaptors interact, and the activation of distinct signaling pathways follows. MyD88 is utilized by all other TLRs except TLR3, whose sole adaptor is TRIF. MyD88 signaling platform involves the recruitment of multiple protein kinases including IL-1 receptor-associated kinases, TNF receptor-associated factors, and transforming growth factor β–activated kinase 1. The signaling events lead to the activation of

Table 1. PRRs and their bacteria/virus –derived PAMPs

	PRR	PAMPs	Origin of the ligand	Site of recognition
TLR	TLR1-TLR2	triacyl lipopeptides	bacteria	cell surface
	TLR2-TLR6	diacyl lipopeptides	mycobacteria	cell surface
		lipoteichoic acid	bacteria	
	TLR2	peptidoglycan	bacteria	cell surface
		lipoarabinomannan	mycobacteria	
	TLR3	dsRNA	RNA viruses	endosomes
	TLR4	lipopolysaccharide	bacteria	cell surface
		envelope proteins	viruses	
	TLR5	flagellin	bacteria	cell surface
	TLR7	ssRNA	viruses	endosomes
		RNA	bacteria	
	human TLR8	ssRNA	viruses	endosomes
	TLR9	CpG DNA	bacteria	endosomes
		DNA	DNA viruses	
	murine TLR11	unknown	uropathogenic bacteria	cell surface
RLR	LGP2	RNA	viruses	cytosol
	MDA-5	RNA	viruses	cytosol
	RIG-I	RNA	viruses	cytosol
NLR	NAIP5	flagellin	bacteria	cytosol
	NLRC4	flagellin	bacteria	cytosol
	NLRP1	anthrax lethal toxin	bacteria	cytosol
	NLRP3	muramyl dipeptide	bacteria	cytosol
		RNA	bacteria, viruses	
		toxins	bacteria	
	NOD1	diaminopimelic acid	bacteria	cytosol
	NOD2	muramyl dipeptide	bacteria	cytosol
		ssRNA	viruses	
Others	AIM2	dsDNA	viruses, bacteria	cytosol
	DAI	dsDNA	viruses, bacteria	cytosol

Abbreviations: AIM2, absent in melanoma 2; DAI, DNA-dependent activator of IRFs; LGP2, laboratory of genetics and physiology 2; MDA5, melanoma differentiation-associated gene 5; NAIP5, neuronal apoptosis inhibitory protein 5; NOD, nucleotide-binding oligomerization domain; NLR, nucleotide-binding and leucine-rich repeat containing gene family receptor; NLRC, NLR family, CARD domain containing; NLRP, NLR family, pyrin domain containing; PAMP, pathogen-associated molecular pattern; PRR, pathogen recognition receptor; RIG-I, retinoic acid-inducible gene I; RLR, RIG-I-like receptor; TLR, Toll-like receptor
Adapted from (23, 24, 38, 45-48)

NF-κB and mitogen-activated protein kinases (MAPKs). TRIF in association with TLR3 or TLR4 complex triggers an alternative pathway whereby interferon regulatory factors (IRFs), in addition to NF-κB, are activated. Consequently, the activation of transcription factors important in the induction of cytokine genes is elicited in the host cell (23, 44).

2.2.2 Cytoplasmic surveillance

NLR family of intracellular receptors is composed of at least 23 members in humans, while 34 NLR-encoding genes exist in the mouse genome. All NLRs have three domains: an N-terminal protein interaction domain, a central nucleotide binding domain, and a C-terminal leucine-rich repeat domain (49). NLRs sense a wide range of microbial PAMPs (Table 1.) and endogenous 'danger' signals. Nucleotide-binding oligomerization domain (NOD) 1 and NOD2 are among the best characterized NLR family members, which detect structural motifs of PG. NOD1 senses *meso*-diaminopimelic acid commonly found in Gram-negative and certain Gram-positive bacteria, whereas NOD2 detects muramyl dipeptide structure of PG abundantly present both in Gram-negative and Gram-positive bacteria (50, 51). Signaling pathways of NOD proteins culminate in the activation of NF-κB and MAPKs (51). This results in the transcriptional upregulation of pro-inflammatory cytokine and host defense genes.

In contrast, upon detection of specific microbial motifs, some NLRs assemble a molecular scaffold termed inflammasome composed of caspase-1 and several adaptor molecules. NLR family, CARD domain containing 4 and neuronal apoptosis inhibitory protein 5 act as sensors for intracellular flagellin (24, 32). NLR family, pyrin domain containing 3 (NLRP3) has been implicated in the detection of bacterial and viral ligands such as derivatives of PG and RNA, as well as endogenous molecules (52, 53). In addition, NLR family, pyrin domain containing 1 detects bacterial toxins (24). These different types of inflammasomes share a common feature: they recruit caspase-1, which promotes processing of pro-IL-1β and pro-IL-18 resulting in the secretion of bioactive cytokines (50).

Interestingly, recent studies have documented the inflammasome activation by a non-NLR receptor absent in melanoma 2, which binds dsDNA by virtue of its HIN200 domain (46-48). Cytosolic dsDNA can also be recognized by DNA dependent activator of IRFs (54), but the biological role of this receptor needs further investigation. The repertoire of nucleic acid sensors is still likely to expand in future.

Three members of RLRs, retinoic acid inducible gene I (RIG-I), melanoma differentiation-associated gene 5, and laboratory of genetics and physiology 2, specialize in cytoplasmic detection of viral RNA (Table 1.). The receptors bear RNA-helicase domains for detection of RNA and tandem caspase activation and recruitment domains (CARDs) for downstream signaling, but laboratory of genetics and physiology 2 receptor lacks CARDs (55). Work by Yoneyama et al. (56) showed that RIG-I lacking CARDs or carrying a mutation in the adenosine triphosphate-binding site of the helicase domain exhibits dominant negative activity. However, ΔRIG-I construct devoid of the helicase domain could induce constitutive downstream signaling, which isa useful property for studying gene activation. After the revelation of RLRs, the interacting adaptor molecule was simultaneously found by four different laboratories, and thus four different names were given to the same molecule (57-60). CARDIF used here stands for CARD adaptor inducing IFN-β. CARDIF localizes to the mitochondria, and mediates signaling to activate NF-κB and IRFs.

Thus, signaling of cytoplasmic detector molecules converges with TLRs on NF-κB and MAPKs, while the activation of IRFs has remained the property of TLRs and RLRs, but not NLRs (Fig. 1). Intriguingly, two reports have now implicated NOD2 in the activation of IRFs and production of type I IFNs (45, 61). It is likely that more such examples exist because the signaling cascades and cooperation of different pathways are only beginning to be understood. Furthermore, the discovery of novel PRRs, especially those of sensing cytoplasmic nucleic acids, will enlighten the current view of pathogen recognition.

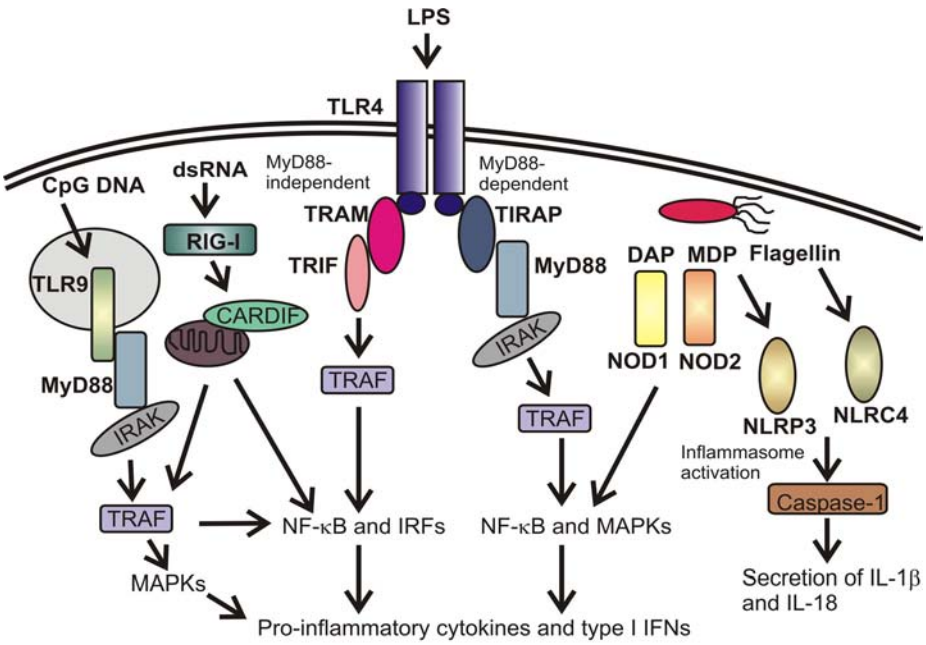

Figure 1. Overview of sensory molecules and their signaling. Representative receptors of cytoplasmic RLR (RIG-I) and NLR (NOD1/2, NLRP3, and NLRC4) families as well as TLRs, located either on the cell surface (TLR4) or endosomes (TLR9), and their signaling are depicted. MyD88 is the key adaptor molecule in all TLR pathways (expect TLR3) coupling IL-1 receptor-associated kinases (IRAKs) and transforming growth factor β-associated kinases (TRAFs) for downstream signaling. Triggering of TLR4 by LPS leads to activation of MyD88-dependent or MyD88-independent pathways involving TIR domain-containing adaptor (TIRAP) and MyD88 or TRAM and TRIF, respectively. RIG-I sensing viral dsRNA interacts with CARDIF localized to mitochondria. TLR and RLR signaling culminates in the activation of NF-κB, IRFs, and MAPK activated transcription factors, which induce the expression of pro-inflammatory cytokines and type I IFNs. Among NLRs, NOD receptors sensing motifs derived from PG, also induce NF-κB and MAPKs. PAMPs liberated from intracellular bacteria can also be detected by other NLR receptors that assemble the caspase-1 inflammasome

2.3 Cytokines

Cytokines are soluble mediators of the immune system that act in autocrine or paracrine fashion as opposed to hormones that have endocrine effects. Cytokines often target several cell types exerting multiple actions, which is referred to as pleiotropy. Moreover, structurally unrelated cytokines can induce similar effects in target cells, thus, redundancy is another characteristic of the cytokine system. Properties of some cytokines are also antagonism or synergism, where two or more

cytokines can inhibit each others function, or act together so that the end result differs qualitatively from that seen with individual cytokines, respectively (62).

Cytokines include IL, IFN, chemokine, colony-stimulating factor, TNF, transforming growth factor β, and growth factor families (62). During microbial infection, panoply of cytokines is released to combat infection. Classical pro-inflammatory cytokines TNF-α, IL-1, and IL-6 are rapidly produced by many cell types in response to microbial infection. Some cytokines with chemotactic properties (chemokines) produced by macrophages and DCs attract other effector cells to the site of infection/inflammation or direct T cell responses thus bridging innate and adaptive immunity. In addition to pro-inflammatory effects, cytokines secreted by phagocytic APCs direct the generation of Th responses. Despite the crucial role of cytokines in resistance to microbial infections, over-production of them may be detrimental to the host. Therefore, the elaborate cytokine system must be tightly regulated.

2.3.1 Cytokines inducing Th1 immunity

Cytokines act as master regulators of immune responses by inducing appropriate Th cell polarization against different types of microbes. Specifically, effective immunity against intracellular bacterial infections requires the differentiation of Th1 cells. The key cytokines regulating Th1 immune responses are IL-12, IFNs and IL-18 (63).

The natural source of IL-12 during microbial infection is the phagocytic cell population. This heterodimeric cytokine consists of p40 and p35 subunits. The IL-12 receptor, composed of two chains – IL-12Rβ1 and IL-12Rβ2 – is mainly found on the cell surface of activated T cells, NK cells, but to some extent also on DCs and macrophages. IL-12 induces the production of IFN-γ by T cells and NK cells, which further activates antimicrobial activities in phagocytes. IFN-γ is the prototypic cytokine produced by cells of the Th1 lineage (18, 63, 64).

The biology of IL-12 is intertwined with that of another IL-12 family member IL-23, as they share the p40 subunit (65), and the IL-12Rβ1 chain of the receptor complex (66). Unique components of the IL-23 cytokine system include p19 subunit (65) and IL-23R (66) for the cytokine and its receptor, respectively. Like IL-12, IL-23 is predominantly produced by phagocytic cells in response to infection and is important in innate immunity. However, instead of inducing Th1 responses, IL-23 appears to play a role in memory T cell responses and in survival and maintenance of Th17 subset (19, 64).

Animal studies in mice have established the role of IL-12, IL-23, and IFN-γ in resistance to many infections, particularly against intracellular bacteria. This is also true in humans, as patients with inherited human immunodeficiencies that affect the IL-12/IL-23–IFN-γ axis due to mutations in genes encoding the cytokine, its receptor, or signaling components, suffer from recurrent infections caused by weakly pathogenic mycobacteria and *Salmonella* species (64, 67-71).

APC-derived IL-12 induces Th1 cells and enhances their IFN-γ production. This can be augmented by function of other cytokines such as IL-18, which is also produced by APCs. IL-12 together with IL-18 increases IFN-γ production by Th1 (72, 73) and NK cells (74). More efficient IFN-γ production can also be mediated by synergism between IL-18 and IFN-α (75, 76). The synergism between cytokines is likely to play a role in enhanced IFN-γ production also in macrophages and DCs, which are not regarded as conventional producers of this cytokine (77, 78).

IL-18 is constantly present in macrophages and DCs as pro-IL-18 form, which is cleaved into biologically active IL-18 by caspase-1. The secretion of mature IL-18 (and IL-1β) is the hallmark of inflammasome activation. IL-18 plays a major role in innate immune responses to pathogens, particularly against intracellular bacteria, whose elimination requires the induction of IFN-γ (50, 79).

2.3.2 Chemokines

The chemokine superfamily with about 50 members in humans can be grouped according to the arrangement of four cysteines in their sequence. Most chemokines fall into the category of CC or CXC classes, in which the cysteines are adjacent or separated with one amino acid, respectively. C and CX3C classes consist of only three chemokines. The G protein-coupled receptors that bind to specific chemokine classes have been named accordingly: CCR1-10 bind CC chemokines, CXCR1-5 bind CXC chemokines, XCR1 binds the only C chemokine lymphotactin, and CX3CR1 binds CX3C chemokines (80-82).

Chemokines and their receptors have a myriad of biological functions including lymphoid organ development, cell recruitment both in homeostasis and inflammation, and development of Th1/Th2 responses. Some functions are shared by many chemokines, whereas others are more specific. This is partly explained by the promiscuity of binding between some chemokine receptors and ligands (80, 81).

Chemokines can be functionally divided into inflammatory and homeostatic groups. Inflammatory chemokines produced during infection recruit effector cells to the

inflammation site and often promote a Th1 cytokine expression profile. Homeostatic chemokines, in contrast, are constitutively produced in various organs including bone marrow, thymus, and secondary lymphoid organs in the absence of apparent infection. Nevertheless, some chemokines may exhibit both inflammatory and homeostatic functions (80).

Chemokines and their receptor system are closely linked to functions of DCs and many recent discoveries in chemokine research have been made in this field. The ability of DCs to switch the pattern of chemokine and chemokine receptor expression during maturation process has been characterized in detail. In a simplified model, immature DCs produce inflammatory chemokines such as CCL5, CXCL10, and CCL20 rapidly in response to infection (83, 84). In addition, they respond to these chemokines via expression of respective receptors. The interaction between CCL20 and its receptor CCR6 on the surface of DCs is considered particularly important for guiding immature DCs to the infection site (83, 84). Moreover, increased secretion of CCL5 and CXCL10 attracts activated memory T cells (85). At later times of infection, DCs coordinately lose their responsiveness to inflammatory chemokines due to the loss of cognate receptor expression while up-regulating the expression of CCR7 (83, 84, 86). This chemokine receptor has two ligands, CCL21 and CCL19, which both attract naïve T cells (87, 88). Mature, but not immature, DCs secrete CCL19 (83, 84), and it has been shown that CCR7 and its ligands play crucial roles in directing mature DCs into T cell zones of lymph nodes, and recruitment of naïve T cells into the vicinity of DCs (83, 89-91).

2.3.3 Interferons

IFNs were originally discovered due to their ability to inhibit viral replication. Three classes of IFNs, based on the receptor complex they use for signaling, are found in humans: type I, type II, and type III IFNs. Signal transduction by all IFNs is mediated via the activation of Janus kinase (JAK) – signal transducer and activator of transcription (STAT) pathway. Previously, only the role of type II IFN was intensively studied in bacterial infections, and the possible role of other IFNs in anti-bacterial immunity is only beginning to be fully acknowledged.

Type I IFNs

Human genome contains genes for IFN-α, IFN-β, IFN-κ, IFN-ε, and IFN-ω. IFN-α subtypes are encoded by 13 functional genes, whereas all other type I IFNs are represented by a single gene. The major subtypes produced during infection are IFN-α and IFN-β. Type I IFNs utilize a receptor consisting of IFNAR1 and IFNAR2

chains, and the signal transduction eventually results in the induction of several hundred IFN-stimulated genes (ISGs) (92-94).

The promoters of type I IFN genes contain multiple regulatory domains that function as binding sites for transcription factors. In the case of IFN-β, these are named as positive regulatory domains (PRDs). PRD-I and PRD-III bind IRF family members, whereas PRD-II and PRD-IV are specific for NF-κB and activator protein 1 (AP-1), respectively. The promoters of IFN-α genes lack NF-κB sites. Upon virus infection, PRDs are known to direct the coordinated recruitment of transcription factors to assemble an IFN-β enhanceosome (95, 96).

Virtually all cells can produce type I IFNs, but the professional cell type secreting vast amounts of type I IFNs particularly in viral infections was identified to be pDC (97, 98). The ability of pDCs to produce high levels of type I IFN is partly due to their strong expression of TLR7 and 9 (27). In other cells, stimulation of TLR3- and TLR4-dependent pathways (utilizing TRIF) may result in type I IFN production (99).

Moreover, TLR-independent systems such as RLRs (56, 100), DNA-dependent activator of IRFs (54), and yet unidentified intracellular sensors can trigger the induction of type I IFNs (101-110). Many of these and other studies have confirmed that also bacterial infection can lead to a robust type I IFN response (38, 101, 103-112). In the search of novel immunoreceptors capable of activating type I IFN response, new bacterial ligands are also being discovered. An example is the bacterial second messenger cyclic-di-guanosine monophosphate that induces type I IFNs via activation of IRFs, NF-κB, and MAPKs (102).

With the exception of pDCs, host cells use an amplification loop strategy to increase the amount of type I IFN. IFN-β and in certain cells, IFN-α4, are synthesized rapidly in response to infection. The small amount of secreted IFN binds to the IFNAR on the cell surface and this autocrine action stimulates the synthesis of more IFN-β and other IFN-α subtypes (113, 114). This feedback loop seems highly important in defense against viral infections. Moreover, evidence from *in vitro* studies suggests that similar positive feedback system operates in *Listeria monocytogenes*-infected macrophages (108, 115) and *Mycobacterium tuberculosis*-infected DCs (112).

The TLR4-TRIF-dependent signaling in response to LPS stimulation leads to the production of type I IFNs, IFN-β representing the major subtype. A subset of genes regulated by LPS stimulation appears to be dependent on type I IFNs. Interestingly, mice deficient in IFN-β production show resistance to LPS-induced endotoxin shock (92, 116, 117). Thus, type I IFNs produced in response to LPS has important effects on the immune system. Moreover, type I IFNs can trigger DC

differentiation/activation as well as influence DC-mediated T cell responses. It is therefore evident that type I IFNs produced during infection provides a critical link between innate and adaptive immune responses (118, 119).

Interestingly, the role of type I IFNs in bacterial infection is multifaceted. They can confer protective effects as is the case in *Salmonella enterica* serovar Typhimurium (120) or *Legionella pneumophila* (103, 121) infection. Type I IFNs are also crucial in host resistance against Gram-positive group B streptococci and pneumococci (122). In Gram-positive *Listeria monocytogenes* infection, however, the production of type I IFNs is detrimental to the host (115, 123).

Type II IFN

Type II IFN or IFN-γ shows no amino acid identity to other IFNs. IFN-γ signaling is transmitted via IFN-γ receptor composed of IFNGR1 and IFNGR2 chains. During infection, the major source of this IFN type is activated T cells and NK cells. IFN-γ acts mainly on macrophages by inducing several antibacterial target genes such as inducible nitric oxide synthase and nicotinamide adenine dinucleotide phosphate-oxidase, which have direct bactericidal effects in cells (92-94).

Interestingly, emerging evidence has demonstrated that IFN-γ can also be secreted by macrophages and DCs (77, 78, 124-126). Most of these studies used different combinations of cytokines or TLR ligands, but reports using live bacteria such as *Listeria* (126) and mycobacteria (124) have revealed the importance of APC-secreted IFN-γ during bacterial infections.

Type III IFN

Human type III IFN or IFN-λ family consists of IFN-λ1, IFN-λ2, and IFN-λ3 (synonyms include IL-29, IL-28A, and IL-28B, respectively) (127, 128). The murine type III IFN system differs from that of humans in the respect that only IFN-λ2 and IFN-λ3 are functional. The gene encoding for IFN-λ1 is a pseudogene in the mouse (129). Type III IFNs mediate their antiviral effects via signaling through a receptor complex composed of IL10R2 and IFNLR1 chains (127, 128). Despite the use of different receptor complex, type I and type III IFNs both activate the formation of interferon stimulated response gene factor 3 (ISGF3), composed of STAT1, STAT2, and IRF9, as well as STAT1 homodimers recognizing IFN-γ activated sequences (127, 129, 130) (Fig. 2). Consequently, the gene expression profile triggered by type I and type III IFNs appears to be practically uniform (131).

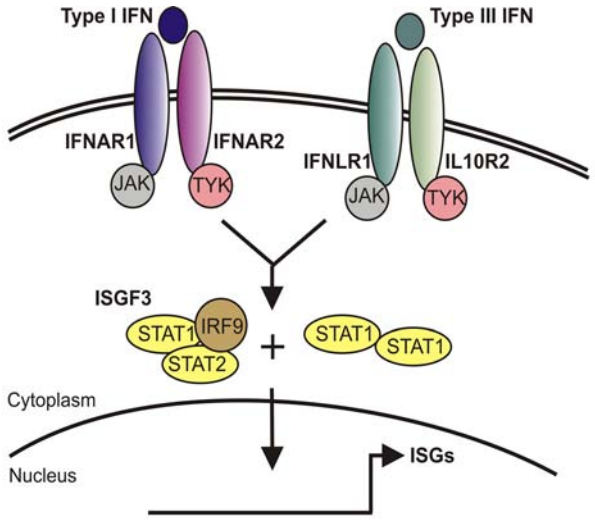

Figure 2. Signaling of type I and type II IFNs. Despite the use of different receptor complex, both IFN types activate JAK-STAT pathway and trigger the induction of ISGF3 and STAT1 homodimers leading to induction of ISGs.

Stimulation of mo-DCs with TLR3/TLR4 agonists or viral infection leads to type III IFN expression (99, 132). In pDCs, type III IFNs are induced upon TLR9 activation (99). In monocyte-derived macrophages, the efficient activation of type III IFN genes in response to TLR3 or TLR4 stimulation requires IFN-α priming (133). The generation of mice deficient in IFNLR1 has suggested a specific role of type III IFNs in TLR3- or TLR9-activated antiviral activity. This study did not find a significant role for IFN-λs in resistance against a number of pathogenic viruses (134). By contrast, another study that used IFNLR1 knock-out mice in *Mx1*$^{+/+}$ background concluded that type III IFNs are important in host resistance against pulmonary influenza A but not hepatotropic virus infection (135).

Unlike the ubiquitously expressed IFNAR, the receptor for type III IFNs displays more restricted expression pattern. The cellular response to type III IFNs is determined by the expression of the IFNLR1 subunit unique to type III IFN signaling (131, 136, 137). In addition to epithelial cells, human blood DCs as well as *in vitro* cultured monocyte-derived macrophages and DCs express high levels of IFNLR1(130, 136).

Like type I IFNs, type III IFNs have been shown to possess immunomodulatory functions some of which have been described in the human system. In particular, IFN-λ1 activates human monocytes/macrophages to produce cytokines (138) and down-regulates Th2-responses initiated by DCs by modulating cytokine secretion (139). When compared to IFN-β, IFN-λ1-treated DCs displayed only a partially

matured phenotype and were shown to specifically induce the proliferation of suppressor T cells expressing Foxp3 (136).

2.3.4 Transcriptional control of cytokine gene regulation

The rapid innate immune response following microbial attack requires that the changes in gene expression are tightly regulated. The most profound regulation of cytokine genes occurs at the transcriptional level. The diversity of transcription factors that operates in gene regulatory networks of immune system is enormous. Here, three classes of transcription factors – IRFs, NF-κB, and MAPK activated AP-1 – are described and outlined in the context of type I IFN gene induction. These transcription factors form an integral part of the enhanceosome, a multiprotein complex that directs the transcription of IFN-β gene.

IRFs

The IRF family of transcription factors comprises nine members. All IRFs contain a conserved DNA binding region that recognizes a consensus DNA sequence termed IFN-stimulated response element (ISRE). They associate with other transcription factors and co-activators the combinations of which vary according to the specific stimulus and determine the outcome of gene expression. Although it is now clear that IRFs are involved in many regulatory events in innate and adaptive immunity, their role is perhaps most carefully characterized in the context of type I IFN gene induction (95, 140, 141).

Multiple IRFs participate in the induction of type I IFN genes (95, 140, 141). However, IRF3 and IRF7 seem to play key roles at least in viral infection (96). IRF3 is constitutively expressed in cells and upon viral infection, becomes phosphorylated, and is transported into the nucleus (142). Phosphorylated IRF3 can form homodimers or it may heterodimerize with IRF7, and in association with co-activators, IRF dimers bind their target DNA sequence. Two protein kinases, Tank-binding kinase 1 (TBK1) and inhibitory κB kinase (IKK) ε, phosphorylate and thereby activate IRF3 and IRF7 (143, 144). IRF7 is present only in small amounts in the absence of infection (with the exception of pDCs). The increased expression of IRF7 depends on formation of ISGF3 complex that is activated in positive feedback phase of type I IFN signaling (113). The ISGF3 complex consists of STAT1, STAT2, and IRF9, and activates ISGs by binding to ISRE sequence (141).

It was previously thought that IRF3 is responsible for the initial activation of IFN-β gene, whereas IRF7 participates in the induction of IFN-α genes in later phases of virus infection (113, 114). However, experiments in virus-infected *Irf7*$^{-/-}$ mice

demonstrated that IRF7 plays a crucial role in the induction of both IFN-β and IFN-α genes, while IRF3 is likely to have a less important role (145). However, IRF3 may be more important in the activation of other cytokine genes such as CXCL10 (117, 146). In addition, if LPS is used to stimulate type I IFNs, IRF3 rules over IRF7 (117, 145).

The first IRF identified, IRF1, was shown to positively regulate IFN-β transcription in original studies (141). However, the findings that the IFN-β enhanceosome did not contain IRF1 (96), and that *Irf1*$^{-/-}$ mice had intact type I IFN induction in virus-infected fibroblasts argued against this (141). Recently, IRF1 has been ascribed a more specific role in TLR-mediated signaling that is functional in myeloid DCs. When induced by IFN-γ, IRF1 could interact with MyD88, and in collaboration with NF-κB, mediated the induction of IFN-β among other genes (147).

Viral infection or TLR7/8 stimulation mimicking virus infection have also been shown to activate IRF5 and thereby contribute to the induction of type I IFNs (148-150). Afterwards, IRF5 was reported to activate the expression of pro-inflammatory cytokines, but not that of type I IFNs, via TLR-dependent pathways (151). Therefore, the role of IRF5 in type I IFN induction needs further investigation.

In contrast to other cell types, pDCs constitutively express high levels of IRF7 (152). It has been shown that pDCs depend on IRF7 for induction of type I IFNs. Moreover, IRF7 interacting with MyD88 retains TLR-ligands in endosomal vesicles for prolonged IFN expression (145, 153, 154). Interestingly, DC-specific mode of type I IFN induction is further governed by IRF8 (155, 156). The stimulatory role of IRF8 in transcription IFN genes was specifically required for the feedback phase of induction (156).

The differential activation of IRFs and their contribution to the induction of type I IFN genes during bacterial infection is far less studied. The most commonly reported IRF to be responsible for IFN activation in bacteria-infected cells is IRF3 (101, 103, 106-108, 110, 112). IRF1 has been implicated in TLR7-dependent bacteria-induced IFN production in conventional DCs, but not in pDCs or macrophages (38). Interestingly, a study performed in human DCs infected with *Mycobacterium tuberculosis* provided correlative evidence for the role of IRF7 (in addition to IRF3) in the expression of type I IFN genes (112). Very recently, *Mycobacterium tuberculosis*-induced IFN induction was depicted to employ IRF5, but not IRF3 or IRF7, in mouse cells (61). Similar contrasting results have been reported from *Listeria monocytogenes*-infected mouse (108, 115) or human cells (157). The two former studies described IRF3 and IRF7 to be important in type I IFN induction, whereas the latter found no role for IRFs. These discrepancies imply that the differential activation of IRFs can be species-specific.

NF-κB

The mammalian NF-κB family includes five members: p65/RelA, p50, RelB, p52, and c-Rel. The Rel homology domain of these proteins mediates binding to DNA, dimerization, and interaction with IκB proteins. Formation of different homo- or heterodimers determines target gene specificity as different NF-κB sites display selectivity in NF-κB dimer usage. Among NF-κB partners, p50 and p52 lack a transactivation domain; thus homodimers of p50 or p52 are mainly repressors of transcription. The prototypical NF-κB complex is composed of p50 and p65. This heterodimer is activated via the classical pathway, which enables a multiprotein IKK complex to trigger the phosphorylation of IκB proteins. In steady state, NF-κB is kept inactive in the cytoplasm by IκB proteins. Only upon stimulus, IκB is degraded and NF-κB translocates into the nucleus. By contrast, the alternative NF-κB signaling pathway results in the formation of RelB-p52 heterodimers. This pathway involves a different IKK complex, and is activated only by few ligands unlike the promiscuously induced classical pathway (158-160).

NF-κB is involved in the transcription of many cytokine genes including IFN-β. Together with IRFs and other co-activators, NF-κB (p50/p65) is part of the IFN-β enhanceosome (96, 161). In the case of IFN-β, each transcription factor class binds onto its respective site in the promoter region. However, NF-κB p65 and IRF3 can also directly interact and function as each others' co-activators for the transcription of IRF- or NF-κB-dependent genes (162, 163).

MAPKs

Three major MAPK cascades operate in mammalian cells: the p38 MAPK, the extracellular signal-regulated protein kinases (ERK), and the c-Jun NH_2-terminal kinases (JNK) (Fig. 3.). MAPKs are activated via phosphorylation by upstream kinases that in turn are activated in response to various extracellular stimuli such as stress, growth factors, and cytokines. MAPKs themselves phosphorylate several downstream molecules including kinases and transcription factors, and hence affect the gene expression (164, 165).

The MAPK activated transcription factors such as AP-1 are involved in the regulation of many cytokine genes. The dimeric AP-1 is composed of members of Jun and Fos families, and binds to AP-1 sites present on target genes (166). For IFN-β gene transcription, AP-1 heterodimer of c-Jun and ATF2 is recruited to the IFN-β enhanceosome upon virus infection (95, 96).

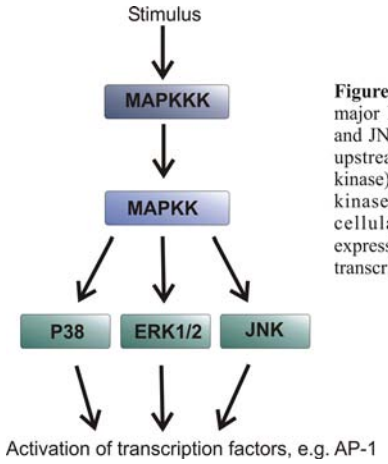

Figure 3. MAPK pathways. Three major MAPK pathways, p38, ERK1/2, and JNK, are activated by the action of upstream kinases MAPKK (MAPK kinase) and MAPKKK (MAPK kinase kinase). MAPKs regulate various cellular activities such as gene expression by phosphorylation of target transcription factors.

2.4 *Salmonella*

2.4.1 Overview of *Salmonella* pathogenesis

Salmonella enterica serovar Typhimurium (*S. typhimurium*) is a common cause of gastroenteritis in humans. However, in mice the same bacterium manifests an illness resembling human typhoid fever, a systemic disease caused by *Salmonella enterica* serovars Typhi or Paratyphi. Thus, *S. typhimurium*-infected mice provide a model for studying systemic *Salmonella* infections. *Salmonella* gastroenteritis infections are usually self-limited in humans and the symptoms include fever, cramping, and diarrhea. In rare cases, bacteremia may develop and lead to severe complications. Subsequent to the actual intestinal salmonellosis, *Salmonella*, among other enteric bacteria, can also trigger an acute inflammatory joint disease called reactive arthritis (167-169).

As a food- and water-borne pathogen, *Salmonella* must traverse the intestinal epithelium to exit the lumen of intestine. This occurs via several routes including penetration of M cells or epithelial cells, and capture of bacteria by DCs. Once across the intestinal epithelium, *Salmonella* interacts with macrophages and DCs which take up the bacteria. Bacteria can also disseminate to mesenteric lymph nodes and other lymphoid sites or reach the blood stream through transportation within phagocytic cells (10, 168).

To resist the defense activities of the host, *Salmonella* has evolved several virulence mechanisms. *Salmonella* pathogenicity islands (SPIs) encode type III secretion systems that deliver virulence factors into the host cell. SPI-1 is activated when *Salmonella* is invading epithelial cells, whereas SPI-2-encoded virulence effectors

are required for the intracellular survival of *Salmonella* inside phagocytes. Moreover, the activity of PhoP/PhoQ, a two-component system, is necessary for the survival of bacteria in the harsh milieu of phagocytes (168).

2.4.2 Interaction of *Salmonella* with phagocytes

Historically, *Salmonella* was considered an facultative intracellular pathogen of macrophages. With the advent of specific tools for phenotypic characterization and culture techniques for DCs, it was demonstrated that also DCs are important havens for *Salmonella. Salmonella* faces its phagocytic hosts once traversed the epithelial cell lining after which murine DCs present in Peyer's patches can take up *Salmonella* (10, 170). Alternatively, CD18-expessing phagocytes provide *Salmonella* a route to bypass the obstacle formed by the epithelium. They can transport *Salmonella* from the gastrointestinal tract to the bloodstream and seed lymphoid sites (10, 171). In addition, Rescigno et al. (172) have described a mechanism through which DCs could capture invasion-deficient *Salmonella* from the lumen of the gut while maintaining the integrity of the epithelium. More recently, a DC subset expressing the chemokine receptor CX_3CR1 was found to sample intestinal microbiota, including *Salmonella,* from the gut lumen (173).

To invade non-phagocytic cells, *Salmonella* induces its own entry via injecting SPI-1 encoded effectors into the cellular cytosol. These bacterial proteins promote polymerization of the actin cytoskeleton and formation of macropinocytic membrane extensions, which results in bacterial internalization (174). Phagocytic macrophages and DCs have an inherent ability to take up microbes via phagocytic receptors. The uptake routes of *Salmonella* in macrophages depend either on the host cell receptors such as complement or Fc receptors or *Salmonella*-induced invasion factors encoded by SPI-1 (175). However, the virulence genes important for the entry of *Salmonella* in macrophages are not required in DCs (176). *Salmonella*-mediated endocytosis in macrophages resembles macropinocytosis, a form of endocytosis allowing the internalization of fluid-phase solute inside large vesicles. Macropinocytosis is dependent on actin polymerization and is found constitutively active in immature DCs. Chemicals that block actin polymerization inhibit both phagocytosis and macropinocytosis, and therefore inhibit bacterial internalization (168, 177, 178).

The interaction of *Salmonella* and the phagocyte leads to the formation of a specialized vacuole called *Salmonella* containing vacuole (SCV) (179), which protects the bacteria from antibacterial activities of the host. The vacuolar compartment and SPI-2-encoded effectors inhibit the fusion of *Salmonella*-

containing phagosomes with lysosomes (180, 181). In addition to macrophages, the intracellular survival of *Salmonella* is promoted in murine DCs (170, 176, 182, 183). Interestingly, *Salmonella* strains attenuated in survival within macrophages can still persist in DCs (176, 182). Moreover, although phagocytes actively induce antibacterial defense mechanisms, there is evidence that *Salmonella* kills DCs involving host caspase-1 activation (184, 185), a phenomenon first observed in macrophages (186).

Salmonella also exploits the migratory capacity of phagocytes to establish a systemic infection. CD18-expressing phagocytes can transport bacteria into the liver and spleen (171). Intestinal lamina propria DCs that sense *Salmonella* flagellin are suggested to contribute to systemic infection as they transport bacteria preferably to mesenteric lymph nodes (33). *In vitro* and *in vivo* evidence implies that migration of *Salmonella*- containing DCs depends on the chemokine receptor CCR7 and its ligands CCL19 and CCL21 (187, 188). On the other hand, this migratory event is required for the induction of T cell responses by DCs. Indeed, it is now evident that DCs are highly important in generation of anti-*Salmonella* immunity (10).

2.5 Sendai virus

Sendai virus or murine parainfluenza type I belongs to the genus *Respirovirus* of the family *Paramyxoviridae*. It is considered non-pathogenic in humans, but causes severe respiratory infections in mice. The virus particle is enveloped and contains the genetic material as negative-stranded ssRNA. The virus genome is composed of the nucleocapside, phospho-, matrix, fusion, haemagglutinin-neuraminidase, and large protein genes (189, 190).

Many intracellular signaling cascades such as TLR- and RLR-dependent pathways are activated in Sendai virus-infected cells. The replication of Sendai virus occurs in the cytoplasm, and the dsRNA formed in the replicative stage serves as a major PAMP recognized by intracellular PRRs. Sendai virus induces an efficient antiviral response including type I IFN production making Sendai virus an excellent tool to study the activation of innate immune mechanisms in molecular detail (132, 190, 191).

3 Aims of the study

Cytokines play an important role in regulating host immune responses during infection. The starting point of this study was that DCs of mouse origin had recently been characterized as host cells for *S. typhimurium*. However, only little was known about the immune responses initiated in *Salmonella*-infected DCs. Thus, cellular responses of macrophages and DCs, in particular the pattern of cytokine production, to *S. typhimurium* were compared. Another focus of this work was to unravel the regulatory events controlling the expression of cytokine genes of CCL19 and type III IFNs in human DCs.

The specific objectives of this study were:

1. To analyze the activation of immune responses in *S. typhimurium* –infected DCs using macrophages as a reference point.

2. To characterize the requirements for transcriptional induction of CCL19 gene in *S. typhimurium* –infected DCs.

3. To study the regulation of most recently identified IFN family genes, type III IFN, and compare their induction with those of type I IFN genes.

4 Material and methods

4.1 Cell culture

4.1.1 Monocyte-derived macrophages and dendritic cells (I-IV)

Leukocyte-rich buffy coats were obtained from Finnish Red Cross Blood Transfusion Service. To minimize inter-individual variation, each experiment was performed with cells of three to four donors, which were pooled upon collection in majority of cases to obtain enough material for RNA and protein studies. Peripheral blood mononuclear cell (PBMC) population was first separated by Ficoll (Amersham Biosciences) gradient centrifugation. The mononuclear cell layer was collected and washed with PBS.

For macrophage differentiation, monocytes were allowed to adhere onto cell culture plates (1 h at +37°C), and non-adherent cells were removed with thorough washing with PBS. Macrophages were cultured in serum-free macrophage medium (Gibco) supplemented with antibiotics (penicillin 0.6 μg/ml, streptomycin 60 μg/ml) and 10 ng/ml GM-CSF. The medium was replaced every two days. After six to seven days of culture, macrophages were fully differentiated and used in experiments and the medium was replaced to RPMI-1640 (Sigma-Aldrich) supplemented with antibiotics, 2 mM L-glutamine, 20 mM HEPES, and 10% fetal calf serum (FCS; Integro).

For DC differentiation, monocytes were further purified by Percoll (Amersham Biosciences) gradient centrifugation and magnetic depletion of contaminating T and B cells. Monocytes were then allowed to adhere onto cell culture plates (1 h at +37°C), and non-adherent cells were removed with thorough washing with PBS. DCs were cultured in RPMI-1640 medium supplemented with antibiotics, 2 mM L-glutamine, 20 mM HEPES, 10% FCS, 10 ng/ml GM-CSF, and 20 ng/ml IL-4. Fresh medium was added every two days. DCs were used in experiments after 6-7 days of culture.

4.1.2 Cell lines (II,IV)

The human embryonic kidney (HEK)-293 cell line (American Type Culture Collection; ATCC CLR1573) was maintained in continuous growth in Eagle-MEM (Sigma-Aldrich) supplemented with antibiotics, L-glutamine, and 10% FCS.

4.2 Microbes

4.2.1. *Salmonella enterica* serovar Typhimurium (I-III)

Salmonella enterica serovar Typhimurium ATCC 14028 strain was stored in the presence of 15% glycerol at -70°C. *S. typhimurium* was inoculated onto Luria agar and grown overnight at 37°C. One colony was inoculated in Luria broth and grown overnight at 37°C with shaking. The culture was then diluted 1:100 in Luria broth and grown for 2.5 h as above to obtain bacteria in a logarithmic growth-phase. Bacteria were collected by centrifugation and washed with PBS. The multiplicity of infection (MOI) was adjusted by reading the culture density at the optical density of 600 nm. The MOI was confirmed by plating serial dilutions onto Luria plates.

4.2.2. *Streptococcus thermophilus* (III)

Streptococcus thermophilus was obtained from Valio research centre (Helsinki, Finland) and stored in skimmed milk at -70°C. *S. thermophilus* was inoculated onto M17 agar (Lab M, Topley House) supplemented with 20 g/L D (+) lactose monohydrate (J.T. Baker B.V.) and grown overnight at 37°C. One colony was inoculated in M17 broth (Difco) containing 20 g/L lactose and grown overnight. *S. thermophilus* was further passaged by 1:30 dilution to fresh M17 broth containing lactose. After 17 h of culture, bacteria were in late logarithmic growth phase. The desired amount of bacteria was collected by centrifugation. The number of bacteria was determined microscopically in Petroff-Hauser chamber or by plating serial dilutions onto M17 agar plates for viable count calculation.

4.2.3. Sendai virus (II-IV)

Sendai virus (Cantell strain) was cultured in embryonated chicken eggs and used from stocks stored at -70°C. The hemagglutination titer of Sendai was 4096 and the infectivity of the stock in DCs was $6x10^9$ pfu/ml.

4.3 Reagents

4.3.1 Purified cytokines and antibodies (I, II, and unpublished)

The experimental use of purified human cytokines and neutralizing cytokine-specific antibodies raised in animals is outlined in Table 2.

Table 2. Cytokines and antibodies used in this study

	Concentration used	Reference/Source	Used in
Purified cytokines			
IFN-α	100 IU/ml	Finnish Red Cross	I
IFN-β	100 IU/ml	Schering-Plough	unpublished
IFN-γ	100 IU/ml	Finnish Red Cross	I
IFN-λ1	10 ng/ml	ZymoGenetics	unpublished
IL-1β	10 ng/ml	R&D Systems	II
IL-12	10 ng/ml	R&D Systems	I
IL-18	30 ng/ml	Biosite	I
TNF-α	5-10 ng/ml	R&D Systems	I, II
Antibodies			
anti-IFN-α	1200 neutralizing U/ml	(192)	I
anti-IFN-β	80 neutralizing U/ml	(192)	I
anti-IL-12	1 μg/ml	R&D Systems	I
anti-IL-18	1:200 dilution of antisera	(193)	I

4.3.2. TLR ligands (I, III)

S. typhimurium ATCC 14028 smooth LPS (TLR4 agonist) preparation was from Sigma. Purified FliC, flagellin protein (TLR5 agonist) purified from *S. typhimurium* ATCC 14028, was purchased from Alexis Biochemicals.

4.3.3. Pharmacological inhibitors (II, III)

Commercial inhibitors were used to inhibit cellular processes as specified in Table 3.

Table 3. Chemically synthesized inhibitors used in this study

	Concentration used	Cellular target	Used in
Cell signaling inhibitors			
DEM[1]	5, 20, or 100 μM	NF-κB	II
PDTC[1]	5, 20, or 100 μM	NF-κB	II, IV
PD98059[2]	50 μM	ERK MAPK	II, IV
SB202190[2]	10 μM	p38 MAPK	II, IV
SP600125[1]	10 μM	JNK MAPK	IV
Ly294002[2]	50 μM	PI3K	IV
CsA[1]	1 μg/ml	NFAT	II
Other inhibitors			
CHX[3]	30 μg/ml	de novo protein synthesis	IV
dynasore[3]	20-120 μM	dynamin (194)	IV

Abbreviations: CHX, cycloheximide; CsA, cyclosporin A; DEM, diethyl maleate; PDTC, pyrrolidine dithiocarbamate; PI3K, phosphoinositide 3-kinase; NFAT; nuclear factor of activated T cells
Purchased from [1]Alexis Biochemicals, [2]Calbiochem, [3]Sigma

4.4 mRNA expression analysis

4.4.1 Northern blot analysis (I, II, IV)

Total cellular RNA was isolated by the guanidium isothiocyanate/cesium chloride method (I) or by using an RNA purification kit (Qiagen; II, IV). Samples containing equal amounts (10 μg) of RNA were size-fractionated on 1% formaldehyde-agarose gels and transferred onto Hybond-N nylon membranes (Amersham Biosciences). Membranes were hybridized with cDNA probes labeled with α-^{32}P-adenosine triphosphate (3000 Ci/mmol, Amersham Biosciences) by random priming. The membranes were hybridized in Ultrahyb buffer (Ambion), washed in 1 x saline sodium citrate/0.1% sodium dodecyl sulphate (SDS), and exposed to Kodak films at -70°C with intensifying screens. To control equal RNA loading, ribosomal RNA was visualized with ethidium bromide staining, or membranes were hybridized with a β-actin probe.

4.4.2 Quantitative RT-PCR (III, IV)

Total cellular RNA was isolated by using an RNA purification kit (Qiagen) including DNase I treatment. RNA was synthesized into cDNA by TaqMan Reverse Transcriptase kit (Applied Biosystems). The cDNA samples were then amplified in TaqMan Universal PCR master mix buffer (Applied Biosystems) using commercial gene expression assay mix oligonucleotides from Applied Biosystems. The data was normalized to housekeeping β-actin gene (III) or 18S rRNA (IV).

4.5 Protein analyses

4.5.1 Cytokine-ELISA assays (I, II, III)

Cell culture supernatants of different donors were kept separate and stored at -20°C. Cytokine levels from cell culture supernatants were analyzed by antibody pairs and standards from BD Pharmingen, R&D Biosystems, Biosite, and PBL Biomedical Laboratories as indicated in the original publications. All the procedures are based on sandwich enzyme-linked immunosorbent assay (ELISA). IL-12 and IL-10 cytokine measurements presented in 5.1.4 as unpublished data were performed using FlowCytomix bead assay by Bender MedSystems.

4.5.2 Western blotting (II-IV)

Protein lysates were stored at -70°C in the presence of 0.5 mM DTT, 1 mM Na_3Vo_4, and a protease inhibitor mixture. Equal amounts of whole cells or nuclear extracts were separated on SDS- polyacrylamide gel electrophoresis using the Laemmli buffer system. Proteins were transferred onto Immobilon-P membranes (Millipore). Detailed information about the antibodies used for immunoblotting can be found in the Material and Methods section of the original publications. The protein bands were visualized using the enhanced chemiluminescence system (Amersham Biosciences).

4.5.3 Flow cytometry (I, III)

Cells were collected as pooled or donor-separated, washed with PBS, and fixed with paraformaldehyde for 15 min. The cells were stored in 2% FCS/PBS at 4°C. The

expression of cell surface markers on DCs was analyzed using FITC- or PE-conjugated antibodies and appropriate isotype controls. Samples were analyzed with FACScan or FACS Canto II flow cytometer and analyzed with Cell Quest or FACS Diva software, respectively, all provided by Becton Dickinson.

4.6 Transcription factor binding analyses

4.6.1 *In silico* analyses (II, IV)

Promoter regions of human CCL19 and IFN-λ1-3 were analyzed for transcription factor binding sites using freely available softwares such as Vector NTI Suite (Invitrogen), MatInspector (Genomatix), and Transcription Element Search System (http://www.cbil.upenn.edu/cgi-bin/tess/tess). The searches were focused on putative NF-κB or ISRE/PRD sites that could potentially bind NF-κB or IRFs, respectively.

4.6.2 DNA affinity binding method (II, IV)

Synthesized biotinylated DNA oligonucleotides containing the putative transcription factor binding site in the middle were ordered from DNA technology. Equal amounts of nuclear extracts were incubated for 2 h at 4°C with streptavidin-agarose beads (Pierce) coupled to annealed oligonucleotides. After washing of unbound proteins, the oligonucleotide-bound proteins were released in SDS sample buffer and boiled. Equal aliquots were run on SDS-polyacrylamide gel electrophoresis gels and analyzed by Western blotting.

4.7 Transfections (II, IV)

HEK-293 cells were seeded at optimal confluence one day prior to transfection. The reporter and expression plasmids were introduced into HEK-293 cells with FuGENE6 (Roche; II) or TransIT (Mirus; IV) reagent. Renilla luciferase plasmid was used to control transfection efficiency. Total amount of DNA was adjusted with an empty expression vector. In some experiments, cells were infected with Sendai virus. Luciferase reporter assays were performed with a Dual Glo kit (Promega) according to manufacturer's instructions and luminescence was detected with Victor multilabel reader (Wallac).

4.8 Gentamicin protection assay (I, III)

Prior to infection, monocyte-derived macrophages and DCs were cultured for 2 h in antibiotic-free RPMI-1640 medium supplemented with glutamine, HEPES, and 10% FCS. In some experiments, cells had been pre-treated with IFN-γ and/or TNF-α for 16 h (I). Dynasore used in IV was added to cells 10 min before infection. Cells were infected with logarithmic growth phase *S. typhimurium* at a MOI of ~1. Infection was allowed to last 1 h, after which non-internalized bacteria were killed with 100 μg/ml gentamicin-containing medium for additional 1 h. The bacterial uptake was determined at 2 h, whereas for later time points studying the intracellular fate of bacteria, the cells were maintained in the medium containing 20 μg/ml gentamicin. The number of intracellular bacteria was determined at various time points; after thorough washing with PBS, host cells were lysed with 0.2% Triton-X-100 and bacteria released from cell interiors were cultured onto Luria agar to assess colony forming units.

5 Results and discussion

5.1 Interaction of *S. typhimurium* with human APCs

5.1.1 Uptake and intracellular survival of *S. typhimurium* (I)

The ability of *Salmonella* to survive and replicate within phagocytes is an important premise for systemic infection and involves the activation of SPI2-encoded effectors (168). When this study was commenced, reports had addressed the intracellular survival of *Salmonella* in murine DCs (170, 176, 182, 183). Furthermore, Dreher et al. (195) had demonstrated the intracellular persistence of *Salmonella* in human DCs.

We compared the efficiency of bacterial uptake and the ability of *S. typhimurium* to survive in human monocyte-derived macrophages and DCs. Using gentamicin protection assay, bacterial uptake was determined as the percentage of inoculated bacteria surviving antibiotic treatment. In addition, the intracellular fate of bacteria was followed at various time points. Bacterial uptake by macrophages and DCs occurred at a similar range (2-3% of inoculated bacteria) (Fig. 6 in I, top graphs). In contrast to macrophages, viable counts of *Salmonella* in DCs showed a gradual decline already during the first 24 h. Neither cell type could completely eliminate the bacteria during the three-day follow-up (Fig. 6 in I, second graphs from top).

Dreher et al. (195) reported that human DCs harboring *S. typhimurium* for prolonged periods of time (beyond 24 h infection) were shown to undergo extensive apoptosis or necrosis thus interfering with quantification of intracellular bacteria. However, in that particular study cells were infected with MOI of 25, while we used MOI 1 in our experiments to avoid bacteria-induced cytotoxicity. The magnitude of *Salmonella*-induced host cell death correlates with the MOI used and represents one strategy the bacterium uses against host defenses (184-186).

Next, we analyzed whether antimicrobial activities and thus intracellular killing of bacteria could be induced by pre-treatment of cells with IFN-γ, TNF-α, or their combination (196, 197). In our model setting, cytokine pre-treatment did not seem to affect bacterial killing (Fig. 6 in I, three bottom graphs). By contrast, cytokine pre-treatment decreased bacterial uptake when compared to cells without priming (Fig. 6 in I, top graphs). This was most pronounced in DCs treated with IFN-γ and TNF-α; cytokines that will induce maturation of DCs resulting in decreased endocytosis capacity. Contrary to our results, Rescigno et al. (183) showed that IFN-γ pre-treatment enhances killing of *Salmonella* particularly in bone marrow-derived

macrophages. This could simply indicate differences between cells of human and mouse origin responding to *Salmonella* infection.

5.1.2 Production of pro-inflammatory and Th1 type cytokines (I)

During bacterial infection, phagocytic APCs act at the interface of innate and adaptive immunity. The key regulators of immune responses are cytokines – their production modulates the outcome of the immune response and is crucial for the host to resist microbial infections. Early work had demonstrated that besides macrophages, also DCs respond to *Salmonella* infection with the production of cytokines (178, 182, 198). In particular, *Salmonella*-infected murine DCs were reported to produce TNF-α, IL-1, IL-6, and IL-12 (178, 182, 198). Besides studies by Dreher et al. (184, 195), which reported TNF-α, IL-10, IL-12, and IL-18 production in *Salmonella*-infected human mo-DCs, only limited data on the interaction of *Salmonella* with DCs of human origin was available.

Therefore, we considered it important to compare *Salmonella*-induced cytokine production in human macrophages and DCs. To study this, we stimulated monocyte-derived macrophages and DCs with *S. typhimurium* and detected cytokine expression at mRNA or protein level. Both cell types induced the expression of TNF-α, IL-12 p35, IL-12 p40, IL-18, and IFN-γ genes (Fig. 2 in I). In accordance with results by Dreher et al. (184, 195), we also detected the production of TNF-α, IL-12 (p70 form), and IL-18, albeit the level of IL-18 in DCs was modest as compared to macrophages (Fig. 3B in I). IL-12, IL-18, and IFN-γ are known to favor the induction of Th1 responses that are effective against intracellular pathogens such as *S. typhimurium* (18, 64, 199).

An interesting novel observation was that IFN-γ protein accumulated in the cell supernatants of *Salmonella*-infected macrophages and DCs at late times of infection (24 and 48 h). This suggested its production to be regulated by other cytokines. As IL-18 together with IL-12 or type I IFNs is known to induce synergistic production of IFN-γ in NK and T cells (72-76), the conventional producers of this type of IFN, we tested whether IFN-γ production in macrophages and DCs was dependent on other Th1 type cytokines. By using neutralizing antibodies against IFN-α/β, IL-12, and IL-18 in different combinations, a significant decrease in IFN-γ production by *Salmonella*-infected macrophages was seen when all the tree antibodies were combined (Fig. 4A in I). In DCs, however, anti-IL-18 alone or combined with anti-IL-12 could significantly reduce IFN-γ production (Fig. 4A in I). Consistently, when cells were stimulated with purified IFN-α, IL-12, or IL-18 preparations, macrophages required all three cytokines for efficient IFN-γ production. In DCs,

instead, the addition of IL-12 and IL-18 was sufficient for maximal IFN-γ production (Fig. 4B in I). We concluded that in DCs, the contribution of IL-12 and IL-18 to IFN-γ production is more important than in macrophages. It remains possible, however, that other cytokine-dependent or –independent mechanisms are involved in regulation of IFN-γ production in this experimental setting, as IFN-γ levels produced in response to bacteria were at least ten-fold higher as compared with the levels obtained with cytokine stimulations (Figs 3B and 4B in I).

As mentioned above, DCs or macrophages were not considered typical sources of IFN-γ, although reports with such findings existed before ours (77, 78, 125, 126). Of note, later work in our laboratory has shown that mo-DCs can induce the production of IFN-γ even in response to Gram-positive, probiotic bacteria (200). Thus, DCs can respond to bacterial infection by producing IFN-γ, and this is not restricted to infection with *S. typhimurium.* This early source of IFN-γ by DCs and macrophages is believed to facilitate the innate responses against microbes prior to adaptive T cell-mediated immunity. IFN-γ produced in autocrine prime loop by myeloid cells increases their production of IFN-γ and IL-12, which serve as signals for Th1 cell differentiation (63).

5.1.3 Production of chemokines (I, II)

Chemokines are classified as cytokines that have chemotactic properties and thus are involved in regulating cellular traffic. At the time this study was initiated, no data on *Salmonella*-induced chemokine expression in human DCs were available. We compared the induction of CCL5, CCL19, CCL20, and CXCL10 genes in *Salmonella*-stimulated macrophages and DCs. The major finding from the Northern blot and ELISA data (Figs 2 and 3A in I) was the preferential production of CCL19 or CCL20 by DCs or macrophages, respectively.

Upon maturation, DCs are known to modulate the expression of chemokines such as CCL19 and CCL20, and their cognate receptors (83, 84, 86). Furthermore, the interplay of CCL19 and its receptor CCR7 is crucial for the migration of DCs into the lymph nodes as well as for the recruitment of naïve T cells into the vicinity of activated DCs (83, 87-91), which leads to the initiation of adaptive immune responses. Although not investigated by us, it is reasonable to assume that CCL19 plays an important role in the biology of *Salmonella* infection. Upon up-regulation of CCR7, mature DCs containing *Salmonella* have been reported to migrate towards CCL19 and CCL21 *in vitro* (187). Moreover, in an adoptive transfer model, transportation of *Salmonella* to the lymph nodes occurred within DCs but not macrophages (188).

Again, the production of CCL19 was not exclusive to *Salmonella* infection as increased levels of this chemokine were also detected in response to infection with Sendai virus (Fig. 1 in II). In an attempt to recapitulate the production of CCL19 with addition of purified cytokines (Fig. 6 in II), we noted that live bacteria were superior inducers of CCL19 compared to stimulation with cytokines or Sendai virus.

5.1.4 Phenotypic maturation of DCs (I, III)

Transformation of an antigen-capturing DC into an efficient APC is collectively referred to as DC maturation. The modulation of cell surface molecules to enable antigen presentation and interaction of DCs with T cells is one of the major events in the process (14-16), and can be easily detected.

Mo-DCs were infected with increasing doses of *Salmonella*, and the expression of CD80, CD83, CD86, and HLA-II was determined by flow cytometric analysis. Compared to unstimulated cells, infected DCs showed up-regulated levels of all the studied molecules (Fig. 1 in I). Even the lowest bacteria/host cell ratio (0.3:1) sufficed at inducing the up-regulation of these marker proteins. Furthermore, up-regulation of CD86 was detected also in response to *Salmonella*-derived LPS and flagellin, ligands of TLR4 and TLR5, respectively (Fig. 5A in I). Previously, it had been observed that murine DCs infected with *Salmonella* up-regulate several co-stimulatory and MHC class I and II molecules leading to the presentation of *Salmonella*-encoded antigens to T cells (176, 178, 183, 198). The up-regulation of CD86 had been described in *S. typhimurium* -infected human DCs (195) and also in accordance with our results, flagellin and LPS had been shown to induce the expression of CD80, CD83, CD86, and HLA-II (201). We and others also observed that although flagellin was able to induce the expression of maturation markers, flagellin-stimulated DCs did not produce cytokines such as IL-12 or CXCL10 (Fig. 5 in I) (201, 202). This indicates that there are clear qualitative differences between TLR4- and TLR5-induced signaling.

Salmonella-induced maturation was also analyzed in the context of signaling to type II IFNs (see section 5.1.5). To block dynamin-dependent endocytosis (194) and thus bacterial uptake (Fig. 6 in III), we pre-treated DCs with dynasore and then analyzed the expression of CD86 and HLA-II. Dynasore-treated, *Salmonella*-infected DCs were impaired in the expression of CD86 and HLA-II compared to mock-treated infected cells (Fig. 4A in III). Similar results were obtained when DCs were stimulated with LPS (data not shown). In general, bacterial uptake is not required for phenotypic maturation of DCs. In reference to *Salmonella*-infected DCs, the up-regulation of CD86 and MHC II in the murine cell model does not require bacterial

uptake (203). In the present time, we are unable to provide a definitive explanation for these contrasting results. Dynasore has been reported to inhibit the TRAM-TRIF signaling in LPS-stimulated murine macrophages (204). In addition, TRIF is required for maximal LPS-induced maturation of murine DCs (205). It is therefore reasonable to speculate that dynasore reduces the expression of CD86 and HLA-II due to interference with TRIF signaling also in human DCs.

5.1.5 Induction of type I and type III IFNs (I, III)

Production of type I IFNs is a hallmark of innate immune responses directed not only against viral infections, but also other classes of pathogens (92-94). Bacteria- and virus-induced IFN synthesis share many characteristics, but the analysis of IFNs in bacterial infection is especially warranted by the fact that they seem to exert variable effects on the host immune responses (92).

We chose to analyze the induction of type I IFNs in monocyte-derived macrophages and DCs infected with *S. typhimurium*. We detected a transient IFN-β expression pattern in our Northern blot analysis (Fig. 2 in I) that seemed to be stronger in macrophages. Later, with a more sensitive quantitative RT-PCR technique, we found that the expression of IFN-β was, in fact, induced more efficiently in DCs (Fig. 1A in III and data not shown). The responses to whole bacteria or TLR4 ligand LPS were inseparable. Consistent with previous results by Coccia et al. (99), IFN-α subtypes were induced only weakly (Fig. 1A in III and data not shown).

In a previous report, TLR4 stimulation of DCs induced not only a transient induction of IFN-β, but also that of type III IFNs (99). This prompted us to analyze the expression of recently discovered IFN-λ family members in bacteria-infected cells. Indeed, stimulation of DCs with *S. typhimurium* or TLR4 ligand LPS led to the induction of IFN-λ1 and IFN-λ2/3 mRNAs (Fig. 1A in III). Human IFN-λ2 and IFN-λ3 are almost identical with 96% amino acid similarity (128) and thus they cannot be distinguished at mRNA or protein level by quantitative RT-PCR or ELISA, respectively. Of note, only IFN-λ1 could be measured at protein level (Fig. 1B in III). To compare the ability of *Salmonella* and Sendai virus to induce IFN activity in host cells, human MxA protein expression was detected by Western blotting. Type I and type III IFNs (but not type II IFN) signaling induces the expression of MxA (132, 206). *Salmonella* elicited the production of MxA practically as efficiently as Sendai virus (Fig. 1C in III) confirming the ability of *Salmonella* to induce IFN response in DCs.

To our knowledge, this is the first report showing that type III IFNs can be secreted by bacteria-infected DCs. Moreover, the mRNA induction of IFN-λ1, IFN-λ2/3, and

IFN-β was not blocked by the presence of CHX, which was used to inhibit *de novo* protein synthesis (Fig. 2 in III) indicating that the mRNA induction is a direct consequence of interaction with bacteria. This is also supported by the rapid kinetics of IFN induction (Fig. 1A in III). Analyzing the effects of cell signaling inhibitors suggested that *Salmonella*-induced type I and type III IFN production requires p38 MAPK, JNK MAPK, phosphoinositide 3-kinase, and NF-κB pathways (Fig. 3 in III).

Induction of type III IFNs and bacterial uptake are coupled

Kagan et al. (204) recently demonstrated that inhibition of dynamin-dependent endocytosis by dynasore blocks LPS-induced TRAM-TRIF signaling in murine macrophages. Consequently, the production of cytokines such as IFN-β was impaired (204). Dynasore is a small molecule inhibitor of the guanosine triphosphate hydrolysis activity of dynamin, which has been implicated in multiple forms of endocytosis (194, 207, 208). We analyzed the induction of cytokines in dynasore-pretreated *Salmonella*-infected DCs and found that the mRNA expression and secretion of cytokines IFN-λ1, CXCL10, and IL-6 were greatly impaired compared to cells with mock-pretreatment (Fig. 5A-B in III). In the report by Kagan et al. (204), dynasore interfered with TLR4-mediated LPS endocytosis by inhibiting IRF3 phosphorylation. Interestingly, we found that dynasore also inhibited the induction of CXCL10, and IL-6 when cells were stimulated with *Streptococcus thermophilus*, an innocuous Gram-positive bacterium (Fig. 5A-B in III). IFN-λ1 was not induced by *Streptococcus thermophilus* stimulation.

Interestingly, dynasore did not block the production of IFN-λ1 in Sendai-virus infected cells (Fig. 5C in III), although dynasore inhibited the phosphorylation of IRF3 in response to Sendai virus infection (Fig. 5D in III). This can be explained by the fact that Sendai virus can enter cells without relying on endocytic pathways (209). In addition, other IRFs such as IRF7 could compensate for IRF3 in Sendai-virus induced IFN-λ1 production. Dynasore also inhibited the *Salmonella*-induced phosphorylation of IRF3 (Fig. 5C in III), which is likely due to reduced uptake of live *Salmonella* by dynasore-pretreated DCs (Fig. 6 in III). As dynasore could also impair cytokine production in response to *Streptococcus thermophilus* (Fig. 5A-B in III), which lacks LPS, we hypothesize that dynasore is likely to interfere with pathways beyond TLR4 signaling. To define the specific endocytosis pathways operating in bacterial uptake in DCs, effector molecules necessary to different forms of endocytosis could be targeted by small interfering RNA approach or by using distinct chemical inhibitors.

Type I and type III IFNs may modulate functions of bacteria-infected DCs

To date, no data is available on the role of type III IFNs in bacterial infection. Genetic ablation of IFNLR1, the unique receptor chain for type III IFNs, has confirmed the role of type III IFNs in antiviral immune responses (134, 135). However, considering that instead of three functional type III IFNs in humans, the mouse immune system is deficient in IFN-λ1 (129), these results should be interpreted with caution. Interestingly, type III IFNs have been shown to modulate cytokine secretion of immune cells and thus the guidance of T cell responses (136, 138, 139).

To address the role of type III IFNs in *S. typhimurium* -infected DCs, we treated cells for 20 h with purified preparations of human IFN-β or IFN-λ1 after which DCs were left uninfected or infected with *S. typhimurium*. After 24 h, cell culture supernatants were analyzed for cytokine production and paraformaldehyde-fixed cells were stained with anti-CD86 or anti-HLA-II antibodies and analyzed by FACS. We found that both IFN-β and IFN-λ1 treatment shifted the cytokine balance so that the production of IL-10 was enhanced and the production of IL-12 was decreased when compared to *S. typhimurium* infection alone (Fig. 4, unpublished). Meanwhile, pre-treatment of DCs with IFNs did not alter *Salmonella*-induced up-regulation of CD86 or HLA-II molecules (Fig. 4, unpublished). Along with the preliminary data presented here and other reports performed with human DCs (136, 138, 139), we speculate that type III IFNs may play a role in bacterial infection and influence the outcome of immune responses. IL-12 favors the differentiation of Th1 cells crucial in anti-*Salmonella* immunity. The production of IL-12 is potently inhibited by IL-10 (18), which has anti-inflammatory properties. Although highly speculative, type III IFNs produced by DCs may act in an autocrine or paracrine fashion and shift the cytokine balance of IL-12 and IL-10 towards IL-10 thus guiding protective Th1 responses (in the case of *Salmonella)* to less favorable T cell responses.

The role of DCs, with special reference to DC-derived cytokines, in *Salmonella* infection is depicted in Figure 5.

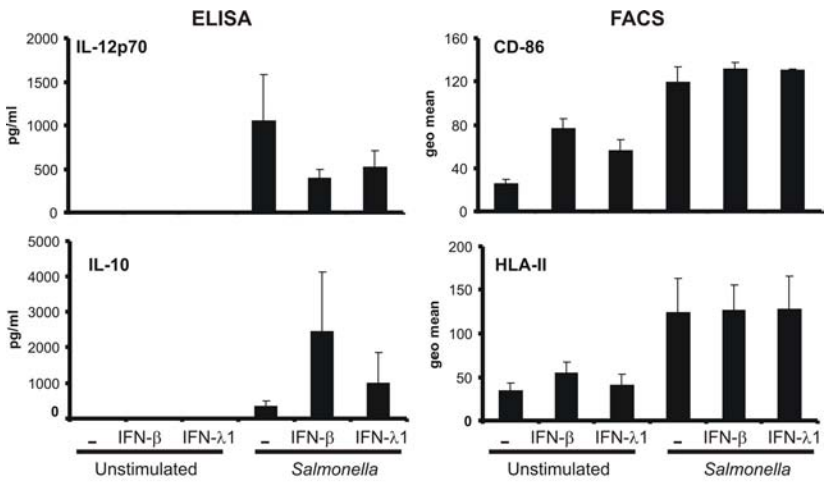

Figure 4. IFN treatment alters the cytokine balance but does not affect phenotypical maturation in *Salmonella*-infected DCs. Cells were mock-treated or pre-treated with IFN-β or IFN-λ1 for 20 h, after which cells were left uninfected or infected with *Salmonella* for 24 h. The levels of IL-12p70 and IL-10 from cell culture supernatants were analyzed by ELISA and the expression of CD86 and HLA-II was determined by FACS.

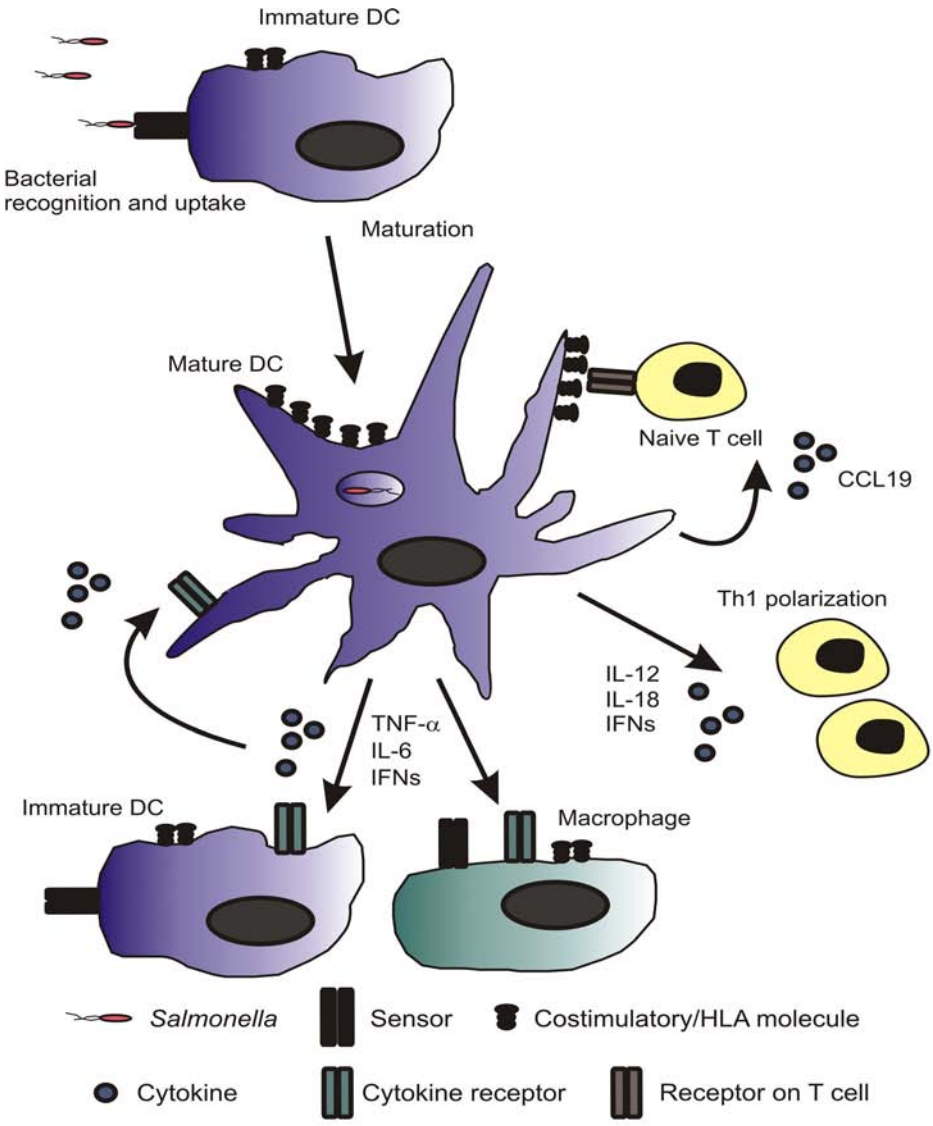

Figure 5. The role of dendritic cells in *Salmonella* infection. Immature DCs sense *Salmonella* through multiple receptors. Immature DCs are characterized by low expression of costimulatory and antigen presentation molecules. *Salmonella* is taken up by DCs and it is able to survive inside the cell in vacuolar compartments. DCs migrating to lymph nodes may therefore contribute to bacterial dissemination. The maturation of DCs is associated with up-regulation of CD80, CD83, CD86, and HLA-II molecules on the cell surface, which facilitates antigen presentation to naïve T cells. CCL19 produced by mature DCs attracts naive T cells into the vicinity of DCs. DC-derived proinflammatory cytokines may act in autocrine fashion or influence neighbouring immune cells, and for example induce the maturation of immature DCs. IL-12, IL-18, and IFNs produced by DCs favor the generation of Th1 responses.

5.2 Transcriptional regulation of cytokine gene expression in DCs

5.2.1 Multiple NF-κB and IRF family transcription factors regulate the expression of CCL19 gene (II)

Although the role of CCL19 in DC biology was clear (see section 5.1.2), limited data on the control of the CCL19 gene expression was available creating a demand for a more detailed analysis of its regulation. Prior to our study, the activation of RelB/p52 dimer through alternative NF-κB pathway had been implicated in the transcriptional control of CCL19 gene (210, 211). Moreover, a histone demethylation event on the CCL19 promoter had been shown to occur concomitantly with RNA polymerase II recruitment in LPS-stimulated DCs (212).

As we had noted that the gene expression of CCL19 is induced in bacteria- or virus-induced DCs (see section 5.1.2 and Fig. 1 in II), we decided to study the regulation mechanisms in detail. First, we employed pharmacological inhibitors targeting p38 and ERK MAPKs, NF-κB, and NFAT to study which signaling pathways are required for the production of CCL19 in DCs. Unfortunately, chemical inhibitors against IRFs were not commercially available. All the inhibitors reduced the mRNA expression of bacteria-induced CCL19 (Fig. 2 in II) at 9 h. At this time point, the inhibitors are likely to act in a specific manner; later time points studied showed inconsistent results (data not shown).

Therefore, another approach was chosen: we characterized the human CCL19 promoter region with computer programs identifying putative transcription factor binding sites. In particular, the proximal promoter was carefully analyzed for the presence of NF-κB and ISRE sites (Fig. 3 in II). Two putative NF-κB sites, NF-κB(1) at -62 to -52 and NF-κB(2) at -363 to -354 nucleotides (relative to the transcription start site) were found. Further upstream at -851 to -842 nucleotides, ISRE consensus element was located. Based on this analysis, we constructed a luciferase reporter construct that would operate under the control of the CCL19 promoter. The 1.5 kilobase promoter fragment contained the three putative transcription factor binding sites discussed above. In addition, promoter constructs with mutated consensus binding sites were created (Fig. 7A in II). Finally, the data was used to construct biotinylated oligonucleotides for DNA affinity binding assays.

To test the functionality of the binding sites, we infected DCs with *S. typhimurium* or Sendai virus, and performed DNA affinity binding experiments with

oligonucleotides containing different consensus sequences derived from the CCL19 promoter. Both NF-κB elements were shown to recruit the binding of classical (p50 and p65) and alternative (p52 and RelB) NF-κB components (Fig. 4 in II). No c-Rel binding could be demonstrated. The components of alternative NF-κB pathway are commonly activated by stimuli such as B cell activating factor, lymphotoxin β, or CD40 ligand (158, 159), but in DCs, the activating signals have been shown to include TNF-α or LPS (210, 211). Moreover, a previous study in a cell line suggested that live *S. typhimurium* or LPS induce the activation of p65, p50, c-Rel, and RelB in DCs (213). In conclusion, DCs can respond to various microbial or danger stimuli by activating the two, the classical and the alternative NF-κB pathways. This allows the formation of distinct sets of NF-κB dimers known to support a different amount of transcriptional activity (160).

In the case of CCL19 ISRE element, we detected binding of multiple IRFs and STATs. Specifically, *Salmonella* induced the binding of IRF1, IRF3, IRF7, IRF9, STAT1, and STAT2 (Fig. 5B in II). Of these transcription factors, the protein expression level of IRF1, IRF7, IRF9, and STAT2 was increased in response to *Salmonella* infection (Fig. 5A in II). Compared to *Salmonella*, the kinetics of Sendai virus-induced binding was generally faster, and phosphorylated form of IRF3 could be easily detected (Fig. 5B in II). STAT1 and STAT2 together with IRF9 form an ISGF3 complex, which binds to ISRE sequences. However, using CCL19 ISRE oligonucleotide in electrophoretic mobility shift assay we failed to detect its formation in microbe-induced DCs (data not shown). Thus, the specific contribution of STATs to regulation of CCL19 remained unknown.

The wild-type and mutant CCL19 promoter constructs were then used to transfect HEK-293 cells. The expression of p50/p65 or p52/RelB dimers could drive the transcriptional activity of CCL19 promoter (Fig. 7B in II). Of the NF-κB elements, the proximal NF-κB(1) seemed to be more important as the NF-κB(2) site mutated on top of the mutated NF-κB(1) did not further reduce the promoter activity (Fig. 7B in II). We also studied the role of IRFs in the activation of CCL19 promoter in a similar assay. IRF1, IRF3, and IRF7, whose binding to CCL19 ISRE had been demonstrated in response to Sendai virus infection (Fig. 5B in II), were transiently expressed in cells along with promoter constructs, after which Sendai virus was added in order to activate the IRFs. IRF1, IRF3, and IRF7, but not IRF5, could induce CCL19 promoter activity, which was inhibited by mutation of the ISRE site (Fig. 7C in II).

To provide insight into the signaling pathways that could induce CCL19 promoter activation, we transfected cells with expression constructs for the upstream transcription factors. The components detecting the cytoplasmic presence of viruses, RIG-I and CARDIF, activated CCL19 promoter transcription (Fig. 8 in II).

Moreover, MyD88 and TRIF, that transmit MyD88-dependent and MyD88-independent signaling, respectively, also mediated transcriptional activity. TLR- and RIG-I -mediated signaling are known to converge at the level of TBK1/IKKε (57-60), and so, overexpression of TBK1 or IKKε could consistently promote CCL19 promoter activation (Fig. 8 in II).

Taken together, the transcriptional activation of the human CCL19 gene requires concerted action of many transcription factors including both classical and alternative NF-κB components, IRFs, and most likely STATs. Our results demonstrating the importance of NF-κB in the activation of CCL19 gene were consistent with previous studies (210, 211). Moreover, a very recent report by Gasparini et al. (214) has suggested that in mature DCs, RelB is only active when it dimerizes with p50 (instead of p52). With a very elegant approach using adenoviral vectors expressing RelB or p50, the authors showed that RelB/p50 preferentially induces CCL19, but not other cytokines and chemokines. The authors further speculated that the NF-κB(1) site analyzed in this study (II), may in fact bind RelB/p50 dimers (214). Indeed, this seems possible considering that preferential activation of RelB/p50 complexes in mature human DCs has been previously reported (213, 215).

5.2.2 The regulation type I and type III IFN genes – common mechanisms underlying (IV)

Type I IFN production is mainly controlled at the transcriptional level (95). For the most part, the biological activities such as antiviral host defenses induced by type I IFNs are shared with type III IFNs (127, 128, 132, 134, 135) – this led us to elucidate the common and disparate mechanisms involved in the transcriptional regulation of type III IFN versus type I IFN genes.

The experimental system to study the regulation of IFN genes was Sendai virus-infected human DCs where robust type I IFN and type III IFN gene expression occurs (Fig. 1 in III) (132). Of note, the expression of IFN-λ1 and IFN-β mRNAs appeared earlier than those of IFN-λ2/3 and IFN-α1 mRNAs (Fig. 1 in III). By using the promoter structure of IFN-β as a reference point, we again used computer programs to identify putative binding sites for IRFs and NF-κB on type III IFN genes. The proximal IFN-λ1 promoter contained at least two ISRE/PRD and two NF-κB elements, whereas only one NF-κB and two ISRE/PRD sites were located in the promoter region of IFN-λ2/3 (Fig. 2 in III and Fig. 6). We tested the functionality of these promoter elements by DNA affinity binding assay, and detected virus-inducible binding of p65, p50, and RelB or IRF1, IRF3 and IRF7 to

the NF-κB or ISRE/PRD sites, respectively (Fig. 3 in III). The binding of IRF8 or p52 remained unchanged in virus stimulation. Previous work in our laboratory had demonstrated that the two distal elements on the IFN-λ1 promoter studied here, ISRE and NF-κB2, were functional in influenza virus-infected DCs (132). Simultaneously with us Onoguchi et al. (216) had described a set of putative IRF and NF-κB binding sites present in human type III IFN promoters. Using HeLa cells, they showed by electrophoretic mobility shift assay that p50/p65 dimers could bind to NF-κB site of IFN-λ1 (216); this site corresponds to NF-κB1 element described by us (Fig. 3 in III).

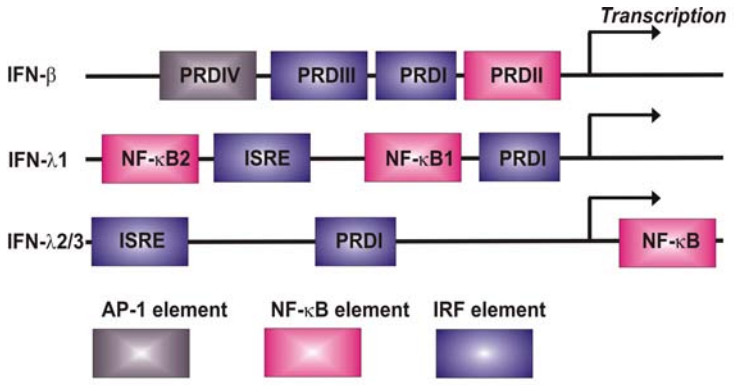

Figure 6. The proximal IFN-β and IFN-λ promoters contain similar transcription factor binding sites. The binding sites on IFN-β promoter are traditionally designated as PRDs. The IRF binding sites on IFN-λ promoters denoted ISREs resemble the closely spaced PRDIII and PRDI of IFN-β, whereas PRDI sequences of IFN-λs are identical to IFN-β PRDI sequence and thus named accordingly. Drawn not to scale.

Similar to studying the regulation of CCL19, we constructed promoter fragments of IFN-λ1 and IFN-λ3 carrying the crucial *cis*-acting elements and compared their activation to much studied IFN-α and IFN-β promoters. First, we focused on IRF1, IRF3, IRF5, IRF7, and IRF8, which are known to positively regulate type I IFN gene induction (95). The differential role of IRFs was revealed by transfection assays in HEK-293 cells. Sendai virus infection greatly increased the promoter activities of IFN-λ1 and IFN-β; this was induced even more by the concomitant expression of IRF1, IRF3, or IRF7 (Fig. 4A in III). By contrast, the activation of IFN-λ3 promoter followed the pattern of IFN-α promoters. Sendai virus infection promoted weak activation of IFN-λ3, IFN-α1, and IFN-α4 promoters, IRF7 being superior to IRF1 or IRF3 at multiplying this effect (Fig. 4A in III). These findings are in agreement with Onoguchi et al. (216) who showed that IRF3 is more efficient at inducing IFN-λ1 than IFN-λ3 promoter activity. Although IRF5 could induce IFN-β or IFN-λ1 promoter activity, it did not promote the activation of IFN-λ3 or IFN-αs (Fig. 4A in III). Indeed, preferential promoter activation of IFN-β, but not

those of IFN-αs, was driven by IRF5 in a similar experimental setting to ours (150). Interestingly, the presence of IRF8 did not increase virus-induced promoter activities (Fig. 4A in III), in effect, IRF8 could down-regulate IRF7-mediated promoter activity by altering the stability of IRF7 (Fig. 5 in III). As IRF8 has been ascribed a positive role in regulating type I gene expression in the mouse system (155, 156), our results may be indicative of certain species-specific differences in IFN gene regulation.

We also analyzed whether overexpression of NF-κB components could stimulate IFN promoters. IFN-α promoters were not sensitive to p50/p65 expression, whereas the promoters of IFN-λ1, IFN-λ3, and IFN-β were greatly induced reflecting the absence and presence of NF-κB elements in the promoter regions, respectively (Fig. 4B in III). Our data suggests that multiple IRF and NF-κB factors can activate type III IFN gene expression. Consistently, Onoguchi et al. (216) reported that mutation of NF-κB and IRF binding sites of IFN-λ1 promoter (corresponding to NF-κB1 and ISRE) impaired viral-induced gene activity, and the effect was more pronounced when both sites were simultaneously disrupted.

Finally, to identify which signaling pathways emanating from pathogen detection could regulate type III IFN expression, we overexpressed molecules involved in TLR and RIG-I signaling. Again, similarities between IFN-λ1 and IFN-β or IFN-λ3 and IFN-αs were detected. ΔRIG-I, CARDIF, IKKε, TBK1, and TRIF promoted higher activation of IFN-λ1 and IFN-β than of IFN-λ3 and IFN-α promoters (Fig. 6 in III). The role of CARDIF, RIG-I, and TBK1 in the induction of type III IFNs had already been suggested by Onoguchi et al. (216). MyD88 serves as a signaling adaptor molecule for type I IFN induction by TLR7, TLR8, and TLR9 (44). Further, MyD88 can interact with IRF1, IRF5, and IRF7, which is required for gene induction (44). Thus, we mimicked TLR7/8 ligation by combining MyD88 expression construct with above IRFs in the presence of Sendai virus infection. MyD88 alone could induce only a moderate activation of IFN-α promoters (Fig. 6B in III). The combination of MyD88 and IRF7 was the only good stimulus for activating IFN-λ3 and IFN-αs, whereas IFN-λ1 and IFN-β were highly responsive when MyD88 was combined with IRF1 or IRF7. Although IFN-α4 and IFN-β promoters were responsive, IFN-λ1, IFN-λ3, and IFN-α1 promoter activities were not induced by MyD88 and IRF5. Thus, we concluded that multiple signaling pathways can lead to type III IFN expression. Specifically, RLR-, TLR3-, and TLR7/8-mediated signaling coinciding at the level of TBK1 and IKKε could lead to induction of type III IFNs.

In addition to the IRF and NF-κB binding sites, the promoter region of type III IFN is likely to contain additional regulatory elements. This is suggested by observations in *Salmonella*-infected DCs where the induction of IFN-λ1 or IFN-λ3 mRNA was

most efficiently blocked by p38 MAPK inhibitor and to a lesser extent by PI3 kinase inhibitor (Fig. 3A in IV). The IFN-λ1 protein level was also reduced by JNK MAPK inhibitor (Fig. 3B in IV). Likewise, inhibition of p38 MAPK in Sendai virus infected DCs impairs the induction of type III IFN genes (data not shown). Interestingly, Thomson et al. (217) demonstrated in their very recent work that the human IFN-λ1 promoter contains four distal NF-κB elements originating from transposition events in addition to the two proximal NF-κB sites used by us. These newly described NF-κB elements together with ISRE were found to be crucial for IFN-λ1 induction in LPS-stimulated DCs (217); whether this applies to other stimuli remains unknown.

In conclusion, the regulation of type III IFN genes is reminiscent of type I IFN gene system where coordinate activation of transcription factors such as NF-κB, AP-1, and IRFs leads to gene induction. Moreover, similarities between the expression kinetics, the magnitude of induction, as well as selectivity in transcription factor usage suggest that the regulation of IFN-λ1 and IFN-λ2/3 resembles the control of IFN-β and IFN-α genes, respectively.

6 Concluding remarks

Upon contact with a microbe, several phenomena including microbial phagocytosis and cytokine production are activated in APCs. The signals leading to the cytokine production are transmitted from PRRs that recognize genetic material and/or structural components of microbes. Macrophages and DCs share the ability to sense surrounding microbes and produce cytokines that regulate both innate and adaptive immune responses. Macrophages operate mainly locally by ingesting and destroying microbial pathogens, whereas DCs, after contacting a pathogen, undergo a process of maturation. The mission of mature DCs is to migrate to local lymph nodes and present antigens to naïve T cells.

This study addressed the activation of innate immune responses primarily in DCs infected with *S. typhimurium*. One of the major findings was that *S. typhimurium* could survive in intracellular compartments of human DCs much like in macrophages. We also found that DCs undergo an efficient maturation process in response to *S. typhimurium* infection or stimulation of cells with bacterial components. *S. typhimurium*-infected macrophages and DCs also produce multiple cytokines including IFN-γ, CCL19 and CCL20, as well as type I and novel type III IFNs.

Another main focus of the work was to provide insight into the regulation of CCL19 and type III IFN genes. Many levels of regulation ensure the control of cytokine gene expression; we chose to focus on the transcriptional regulation of these genes. As a common theme, we observed that the regulation of CCL19 and type III IFNs is elicited by simultaneous and coordinate activation of many signaling pathways. While CCL19 was preferentially regulated by NF-κB, IRF transcription factors had a significant positive regulatory role. In the case of type III IFN genes, IRF3, IRF7 as well as NF-κB, seemed to play a major role in regulating their expression, which was reminiscent of type I IFN regulation.

In addition, we found that type III IFNs are produced not only upon virus infection but also in response to bacterial stimulation in human DCs. These type III IFNs may modulate the function of other immune cells. The fundamental differences of the type III IFN system between human and mouse warrant further research in this field not only *in vitro* but also *in vivo*. It is tempting to speculate that by analogy to production of type I IFNs that can confer harmful or beneficial effects to the host, also type III IFNs may exhibit this dual role *in vivo* depending on the infecting microbe.

Recent decade has witnessed the expansion in our knowledge of host innate immune responses. Understanding the complex signal cascades induced by microbial stimuli and accumulating data of co-operativity of different signaling pathways will provide us a more comprehensive background for development of novel vaccines and pharmaceuticals to treat infections and control host inflammatory responses.

7 Acknowledgements

This work was performed at the Viral Infections Unit at the Department of Vaccination and Immune Protection, National Institute for Health and Welfare, Helsinki. Head of the institute, Professor Pekka Puska, and former head of the Department of Microbiology, Professor Tapani Hovi are thanked for providing excellent working facilities.

Financial support for this thesis was provided by the Sigrid Jusélius foundation and the Microbes and Man research program of the Academy of Finland, which are gratefully acknowledged. Also the Graduate School for Biotechnology and Molecular Biology is acknowledged for the funding and good quality courses during my Ph.D. studies.

The supervisor of this work, Professor Ilkka Julkunen, has always been optimistic and enthusiastic about my projects – I wish to thank him for teaching me how to do good science. His wealth of knowledge in science is something to look up to!

Docents Kaisa Granfors and Petteri Arstila are warmly thanked for their careful review process and valuable comments regarding this thesis. I also wish to thank docent Petteri Arstila and Professor Mikael Skurnik for being active members in my thesis committee group.

I also wish to thank Professor Timo Korhonen and Kaarina Lähteenmäki at the Division of General Microbiology, University of Helsinki for introducing me into the fascinating world of my favorite bacterium, *Salmonella*.

All the co-authors and collaborators are warmly thanked for fruitful teamwork. Everybody in the previous INIM lab deserves big thanks – thank you for teaching me all about innate immunity and how to work with human primary cells. I want to thank Ville for his patience and superb guidance during my first years in the lab – thanks for being there in both scientific and non-scientific aspects! I also want to thank Pamela for her great humor and sewing skills! The former and present senior scientists in our INIM group: Anne, Minja, Jaana, MariS, Maarit, and Pasi are thanked for all their precious advice and helpful discussions during my Ph.D. work. Sinikka and Sanna, thanks for being great company in conference trips and numerous seminar days. Riku and Janne are especially thanked for their skills in making glögi for Christmas parties and Laura for introducing extraordinary combinations for the taste buds in our ‘kahvihuone’! MariA, Sari, Hanna, Johanna,

and Teija – thanks for keeping the lab in order and helping me with miscellaneous things.

The smiley secretaries Kirsi-Mari, Tia, Minna, and Riitta have also made bureaucratic paper work seem somewhat simple – I couldn't have survived without your help. Our IT-help has always been of superior quality thanks to Carina. I wish to thank her also for the numerous Lost series…

My friends outside the lab are also warmly thanked for their support and trust in me. Heli, thanks for staying in Finland – you are the best company for shopping, gym, and having fun in general! You have obediently listened to my complaints about lab experiments gone wrong… Susanna, Annika, Katja – you are all very dear to me no matter where you live! My friends from high school years, Kirsi & Antti, Kirsti & Pauli, Marianne, and Tarja, thanks for being patient with my never-ending PhD work… My microbiologist friends Anni and Jukka – I think we should found our own lab and quit complaining! Tea, you are the master of wine and partying! Aino, and Milla, thanks for all talks over coffees. My Montana friends Saku and Lauri, I am proud we still keep in touch!

I am most grateful to my parents who have always believed in me and known that this work will some day be finished… The little breaks at Valijärvi summer house with my mom Marjatta and dad Rauno have offered an escape from the bustle of city life. My sister Maria and her boyfriend Miska, I love hanging out with you whether it is at summer house having barbecues or at home playing board games. Last but not least, I wish to thank Raine, to whom my heart belongs, for his nerves of steel regarding this last year.

Taija Pietilä

Helsinki, December 2009

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The Journal of Immunology

Multiple NF-κB and IFN Regulatory Factor Family Transcription Factors Regulate CCL19 Gene Expression in Human Monocyte-Derived Dendritic Cells[1]

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CCL19 chemokine has a central role in dendritic cell (DC) biology regulating DC traffic and recruitment of naive T cells to the vicinity of activated DCs. In this study, we have analyzed the regulation of CCL19 gene expression in human monocyte-derived DCs. DCs infected with *Salmonella enterica* or Sendai virus produced CCL19 at late times of infection. The CCL19 promoter was identified as having two putative NF-κB binding sites and one IFN-stimulated response element (ISRE). Transcription factor binding experiments demonstrated that *Salmonella* or Sendai virus infection increased the binding of classical p50+p65 and alternative p52+RelB NF-κB proteins to both of the CCL19 promoter NF-κB elements. Interestingly, *Salmonella* or Sendai virus infection also increased the binding of multiple IFN regulatory factors (IRFs), STAT1, and STAT2, to the ISRE element. Enhanced binding of IRF1, IRF3, IRF7, and IRF9 to the CCL19 promoter ISRE site was detected in *Salmonella* or Sendai virus-infected cell extracts. The CCL19 promoter in a luciferase reporter construct was activated by the expression of NF-κB p50+p65 or p52+RelB dimers. IRF1, IRF3, and IRF7 proteins also activated CCL19 promoter in the presence of Sendai virus infection. CCL19 promoter constructs mutated at NF-κB and/or ISRE sites were only weakly activated. Ectopic expression of RIG-I (ΔRIG-I, CARDIF) or TLR3/4 (TRIF, MyD88, IKKε, or TBK1) signaling pathway components induced CCL19 promoter activity, suggesting that these pathways are important in CCL19 gene expression. Our experiments reveal that expression of the CCL19 gene is regulated by a combined action of several members of the NF-κB, IRF, and STAT family transcription factors. *The Journal of Immunology*, 2007, 178: 253–261.

The Journal of Immunology

IFN Regulatory Factor Family Members Differentially Regulate the Expression of Type III IFN (IFN-λ) Genes[1]

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Virus replication induces the expression of antiviral type I (IFN-αβ) and type III (IFN-λ1–3 or IL-28A/B and IL-29) IFN genes via TLR-dependent and -independent pathways. Although type III IFNs differ genetically from type I IFNs, their similar biological antiviral functions suggest that their expression is regulated in a similar fashion. Structural and functional characterization of the IFN-λ1 and IFN-λ3 gene promoters revealed them to be similar to IFN-β and IFN-α genes, respectively. Both of these promoters had functional IFN-stimulated response element and NF-κB binding sites. The binding of IFN regulatory factors (IRF) to type III IFN promoter IFN-stimulated response element sites was the most important event regulating the expression of these genes. Ectopic expression of the components of TLR7 (MyD88 plus IRF1/IRF7), TLR3 (Toll/IL-1R domain-containing adapter-inducing factor), or retinoic acid-inducible gene I (RIG-I) signal transduction pathways induced the activation of IFN-λ1 promoter, whereas the IFN-λ3 promoter was efficiently activated only by overexpression of MyD88 and IRF7. The ectopic expression of Pin1, a recently identified suppressor for IRF3-dependent antiviral response, decreased the IFN promoter activation induced by any of these three signal transduction pathways, including the MyD88-dependent one. To conclude, the data suggest that the IFN-λ1 gene is regulated by virus-activated IRF3 and IRF7, thus resembling that of the IFN-β gene, whereas IFN-λ2/3 gene expression is mainly controlled by IRF7, thus resembling those of IFN-α genes. *The Journal of Immunology*, **2007, 179: 3434–3442.**