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Publications of the National Public Health Institute Ⓐ 2/2007

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

To be presented with the permission of the Faculty of Biosciences, University of Helsinki, for public examination in the small hall, University Main Building, on 16th of February, at 12 o´clock noon.

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ABSTRACT

Human body is in continuous contact with microbes. Although many microbes are harmless or beneficial for humans, pathogenic microbes possess a threat to wellbeing. Antimicrobial protection is provided by the immune system, which can be functionally divided into two parts, namely innate and adaptive immunity. The key players of the innate immunity are phagocytic white blood cells such as neutrophils, monocytes, macrophages and dendritic cells (DCs), which constantly monitor the blood and peripheral tissues. These cells are armed for rapid activation upon microbial contact since they express a variety of microbe-recognizing receptors. Macrophages and DCs also act as antigen presenting cells (APCs) and play an important role in the development of adaptive immunity. The development of adaptive immunity requires intimate cooperation between APCs and T lymphocytes and results in microbe-specific immune responses. Moreover, adaptive immunity generates immunological memory, which rapidly and efficiently protects the host from reinfection.

Properly functioning immune system requires efficient communication between cells. Cytokines are proteins, which mediate intercellular communication. Immune cells produce inflammatory cytokines rapidly following microbial contact. Inflammatory cytokines modulate the development of local immune response by binding to cell surface receptors, which results in the activation of intracellular signalling and modulates target cell gene expression. One class of inflammatory cytokines – chemokines – has a major role in regulating cellular traffic. Locally produced inflammatory chemokines guide the recruitment of effector cells to the site of inflammation during microbial infection.

In this study two key questions were addressed. First, the ability of pathogenic and non-pathogenic Gram-positive bacteria to activate inflammatory cytokine and chemokine production in different human APCs was compared. In these studies macrophages and DCs were stimulated with pathogenic *Steptococcus pyogenes* or non-pathogenic *Lactobacillus rhamnosus*. The second aim of this thesis work was to analyze the role of pro-inflammatory cytokines in the regulation of microbe-induced

chemokine production. In these studies bacteria-stimulated macrophages and influenza A virus-infected lung epithelial cells were used as model systems.

The results of this study show that although macrophages and DCs share several common antimicrobial functions, these cells have significantly distinct responses against pathogenic and non-pathogenic Gram-positive bacteria. Macrophages were activated in a nearly similar fashion by pathogenic *S. pyogenes* and non-pathogenic *L. rhamnosus*. Both bacteria induced the production of similar core set of inflammatory chemokines consisting of several CC-class chemokines and CXCL8. These chemokines attract monocytes, neutrophils, dendritic cells and T cells. Thus, the results suggest that bacteria-activated macrophages efficiently recruit other effector cells to the site of inflammation. Moreover, macrophages seem to be activated by all bacteria irrespective of their pathogenicity. DCs, in contrast, were efficiently activated only by pathogenic *S. pyogenes*, which induced DC maturation and production of several inflammatory cytokines and chemokines. In contrast, *L. rhamnosus*-stimulated DCs matured only partially and, most importantly, these cells did not produce inflammatory cytokines or chemokines. *L. rhamnosus*-stimulated DCs had a phenotype of "semi-mature" DCs and this type of DCs have been suggested to enhance tolerogenic adaptive immune responses. Since DCs have an essential role in the development of adaptive immune response the results suggest that, in contrast to macrophages, DCs may be able to discriminate between pathogenic and non-pathogenic bacteria and thus mount appropriate inflammatory or tolerogenic adaptive immune response depending on the microbe in question.

The results of this study also show that pro-inflammatory cytokines can contribute to microbe-induced chemokine production at multiple levels. *S. pyogenes*-induced type I interferon (IFN) was found to enhance the production of certain inflammatory chemokines in macrophages during bacterial stimulation. Thus, bacteria-induced chemokine production is regulated by direct (microbe-induced) and indirect (pro-inflammatory cytokine-induced) mechanisms during inflammation. In epithelial cells IFN-α and tumor necrosis factor-α were found to enhance the expression of microbe-detection receptors and components of cellular signal transduction machinery. Pre-treatment of epithelial cells with these cytokines prior to virus infection resulted in markedly enhanced chemokine response compared to untreated cells. In conclusion, the results obtained from this study show that pro-inflammatory cytokines can enhance microbe-induced chemokine production during microbial infection by providing a positive feedback loop. In addition, pro-inflammatory cytokines can render normally low-responding cells to high chemokine producers via enhancement of microbial detection and signal transduction.

Keywords: Immune system, leukocyte, cytokine, bacteria, influenza A virus

TIIVISTELMÄ

Immuunijärjestelmä suojaa elimistöä taudinauheuttajamikrobeilta ja pahanlaatuisilta solumuutoksilta. Elimistön mikrobipuolustukseen osallistuvat seerumin antimikrobiset proteiinit ja erityisesti veren valkosolut. Veren ja kudosten neutrofiilit, monosyytit, makrofagit, sekä dendriittisolut tarkkailevat ympäristöään mikrobien ja muiden taudinaiheuttajien varalta. Nämä valkosolutyypit ilmentävät lukuisia mikrobireseptoreja, joiden avulla ne tunnistavat mikrobirakenteita, kuten bakteerien pinnan lipopolysakkarideja, peptidoglykaania tai virusten kaksijuosteista RNA:ta. Tunnistustapahtuma käynnistää solunsisäisen signaalinvälityksen, mikä johtaa valkosolujen geeniluennan ja sitä kautta toiminnan muutoksiin. Valkosoluaktivaation tavoitteena on pysäyttää infektion leviäminen mahdollisimman nopeasti.

Solujen välinen viestintä on ehdoton edellytys toimivalle immuunijärjestelmälle. Liukoiset sytokiiniproteiinit ovat yksi tärkeimmistä immuunivasteen välittäjäaineista. Sytokiinigeenien luenta käynnistyy nopeasti mikrobikohtaamisen seurauksena. Nämä tulehdustyyppiset sytokiinit, eli pro-inflammatoriset sytokiinit, säätelevät immunivasteen kehittymistä mm. aktivoiden antimikrobisten tekijöiden tuotantoa. Eräs sytokiiniryhmä – kemokiinit - säätelee valkosoluliikennettä tulehdusalueella.

Tässä työssä on vertailtu patogeenisen *Streptococcus pyogenes* ja ei-patogeenisen *Lactobacillus rhamnosus*-bakteerin kykyä aktivoida ihmisen valkosoluja. Tulokset osoittavat, että *S. pyogenes* aktivoi inflammatorisen välittäjäainevasteen makrofageissa ja kaikissa tutkituissa dendriittisolutyypeissä. Ihmisen immuunivaste näyttää siis aktivoituvan tehokkaasti *S. pyogenes*-bakteeria vastaan, mikä osaltaan johtaa tämän bakteerin tuhoamiseen ja infektion leviämisen estämiseen. Toisaalta voimakas välittäjäainetuotanto ja siitä mahdollisesti seuraava ylisuuri immuunivasteen aktivaatio saattaa pahimmillaan johtaa kudostuhoon tai streptokokki-infektion jälkitauteina kehittyviin autoimmuunitauteihin. Ei-patogeeninen, elimistön normaaliflooraan kuuluva maitohappobakteeri *L. rhamnosus* aktivoi makrofagit ja dendriittisolut vain osittain eikä saanut aikaan

inflammatoristen välittäjäaineiden tuotantoa dendriittisoluista. Inflammatorisen vasteen puuttuminen voi osaltaan selittää elimistön ja sen normaalimikrobiston välistä toleranssia ja symbioottista vuorovaikutusta.

Tässä työssä on lisäksi selvitetty pro-inflammatoristen sytokiinien vaikutuksia solujen välittäjäaineiden tuotantoon bakteeri- ja virusinfektiossa. Tulokset osoittavat, että infektion aikana tuotettavat pro-inflammatoriset sytokiinit, erityisesti interferoni (IFN)-α, lisäävät patogeenisen *S. pyogenes*-bakteerin aikaansaamaa välittäjäainetuotantoa, mikä edelleen tehostaa immuunivasteen aktivaatiota. IFN-α:n ja tuumorinekroositekijä-α:n osoitettiin myös lisäävän solujen mikrobitunnistusreseptorien ja signaalinvälityskomponenttien ilmentymistä, minkä seurauksena epiteelisolujen virusvaste tehostui merkittävästi. Tulokset osoittavat, että tehokas immuunivasteen aktivaatio riippuu sekä suorista mikrobi-kohdesolu vuorovaikutuksista että infektion aikana tuotettavien sytokiinien välittämistä signaaleista.

CONTENTS

ABBREVIATIONS

APC	antigen presenting cell
CARD	caspase recruitment domain
CD	cluster of differentiation
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DC-SIGN	DC-specific ICAM3-grabbing non-integrin
dsRNA	double stranded RNA
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
GAS	interferon gamma-activated sequence element
GM-CSF	granulocyte-macrophage colony stimulating factor
IFN	interferon
IKK	inhibitory κB kinase
IL	interleukin
Ipaf	ICE protease-activating factor
IRAK	IL-1 receptor associated kinase
IRF	interferon regulatory factor
ISGF	interferon stimulated gene factor
ISRE	interferon-stimulated response element
Jak	Janus tyrosine kinase
LPS	lipopolysaccharide
LRR	leucine-rich repeat
LTA	lipoteichoic acid
MAPK	mitogen-activated protein kinase

MARCO	macrophage receptor with collagenous structure
mDC	myeloid dendritic cell
MHC	major histocompatibility complex
mo-DC	monocyte-derived dendritic cell
mRNA	messenger RNA
MyD	myeloid differentiation factor
NF-κB	nuclear factor kappa-B
NK	natural killer cell
NLR	NOD-like receptor
NOD	nucleotide-binding oligomerization domain
PAMP	pathogen associated molecular pattern
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
pDC	plasmacytoid dendritic cell
PGN	peptidoglycan
PRR	pattern recognition receptor
RIG-I	retinoid acid-inducible gene I
RIP	receptor interacting protein
rRNA	ribosomal RNA
SARM	Sterile alpha and HEAT/Armadillo motif protein
SR	scavenger receptor
STAT	signal transducer and activator of transcription
TBK	Tank binding kinase
TCR	T cell receptor
TGF	transforming growth factor
Th	T helper cell

TIR	Toll/IL-1 receptor
TIRAP	TIR domain containing adaptor
TLR	Toll-like receptor
TNF	tumor necrosis factor
TRAF	TNF receptor-associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain-containing adaptor inducing IFN-β

LIST OF ORIGINAL PUBLICATIONS

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

I Veckman V, Miettinen M, Matikainen S, Lande R, Giacomini E, Coccia EM, Julkunen I. 2003. Lactobacilli and streptococci induce inflammatory chemokine production in human macrophages that stimulates Th1 cell chemotaxis. J Leukoc Biol. 74:395-402

II Veckman V, Miettinen M, Pirhonen J, Siren J, Matikainen S, Julkunen I. 2004. *Streptococcus pyogenes* and *Lactobacillus rhamnosus* differentially induce maturation and production of Th1-type cytokines and chemokines in human monocyte-derived dendritic cells. J Leukoc Biol. 75:764-71

III Veckman V and Julkunen I. 2006. *Streptococcus pyogenes* induces activation of human plasmacytoid and myeloid dendritic cells. Submitted for publication.

IV Veckman V, Österlund P, Fagerlund R, Melén K, Matikainen S, Julkunen I. TNF-alpha and IFN-alpha enhance influenza-A-virus-induced chemokine gene expression in human A549 lung epithelial cells. 2006. Virology 345: 96-104

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1 INTRODUCTION

Human body is in continuous contact with microbes. Although many microbes are beneficial or harmless to humans, pathogenic microbes are a threat and can cause severe infections. Mechanical shield against microbial invasion is provided by rigid skin and sticky mucosal surfaces. Moreover, secreted antimicrobial compounds, enzymes and low pH offer further protection at these sites. However, these primary defence systems are often inadequate as, during the course of evolution, pathogenic microbes have developed means to overcome these barriers and invade. More efficient and powerful antimicrobial defence is provided by the immune system, which can be functionally divided into two parts. The innate immunity acts rapidly and non-specifically towards foreign components. Adaptive immunity, on the other hand, requires time to develop but it is specific to foreign antigens and generates immunological memory.

The non-specific innate immunity requires coordinated action of several effector cells and serum proteins. Bacterial infections are controlled by phagocytic neutrophils, monocyte/macrophages and dendritic cells, which constantly patrol in blood and tissues and are ready to engulf and destroy microbes. The innate immune defence against intracellular bacteria or viruses, which generally can not be detected by phagocytes, is controlled by natural killer (NK) cells, which detect and destroy infected cells. Recognition and uptake of microbes is based on germ line-encoded pattern recognition receptors (PRRs). These receptors detect conserved microbial structures that are not found in the host. The innate antimicrobial defence is significantly assisted by several serum proteins which detect and bind to microbes thereby promoting the uptake and destruction of microbes by immune cells. A common aim of the innate immunity is to rapidly detect and stop the spread of a pathogen. Several components of the human innate immunity have an evolutionarily ancient background and can be found in invertebrates.

In contrast to the innate immunity, adaptive or acquired immunity is specific to foreign antigens. Adaptive immunity offers pathogen-specific detection and targeted immune response, which is usually effective also against those microbes that may evade innate immune responses. Moreover, the immunological memory offers rapid, specific and efficient immune response upon reinfection. The development of adaptive immunity requires a complex co-operation between antigen presenting cells (APCs) (macrophages, dendritic cells, and B cells) and T lymphocytes.

The key factor of a functional immune system is an effective communication between different cells. Cytokines are secretory proteins that act as messengers

between cells. Cytokines are involved in practically all steps of the immune response by regulating homeostasis as well as inflammatory responses. One class of cytokines - chemokines - has an essential role in regulating the traffic of immune cells. Inflammatory cytokines and chemokines are produced at the early times of microbial infection. The local production of these mediators regulates the development of immune response by recruiting cells to the site of inflammation and by modulating the gene expression of effector cells.

In the present work two key questions were addressed. First, the inflammatory cytokine and chemokine response induced by pathogenic and non-pathogenic Gram-positive bacteria was compared in human macrophages and dendritic cells. Secondly, the role of pro-inflammatory cytokines in the regulation of microbe-induced chemokine gene expression was evaluated in bacteria-stimulated macrophages and virus-infected epithelial cells.

2 REVIEW OF THE LITERATURE

2.1 Effector cells of the immune response

Macrophages and dendritic cells patrol in blood and other tissues and these cells are armed to detect and destroy microbes. An important feature, which distinguishes macrophages and dendritic cells from other phagocytes, is that they are able to process exo- or endogenous antigens and present them to T cells in the context of cell surface major histocompatibility complex (MHC) molecules. Thus, while phagocytic neutrophils primarily act as innate immune cells, macrophages and dendritic cells are also able to initiate adaptive immune responses.

2.1.1 Macrophages

The cells of monocyte/macrophage lineage develop in the bone-marrow from common myeloid progenitor cells. The same myeloid progenitor gives rise to granulocytes, megakaryocytes and erythrocytes. Differentiated monocytes are released from the bone-marrow into blood and in a couple of days they enter tissues and differentiate into macrophages. (Reviewed in 44) Macrophages show remarkable diversity in their phenotype and function depending on their anatomical location. Macrophage differentiation and functional specialization is dependent on the tissue microenvironment, which consists of growth factors, cytokines and other cell types present (44). Different macrophage types are involved in various biological processes ranging from tissue/bone remodeling and wound healing to anti-microbial immune defence (173). Macrophages have three major functions in anti-microbial response. They phagocytose and destroy microbes, produce large amounts of cytokines and chemokines and act as antigen presenting cells.

The first step in antimicrobial defence is to detect foreign structures and distinguish them from the self. For these purposes macrophages express multiple pattern recognition receptors (PRRs) which detect conserved structures found in microbes but that are absent from host cells. Macrophages can phagocytose extracellular microbes by using several receptor systems. Scavenger receptors (SRs), such as SR-A, CD36 and macrophage receptor with collagenous structure (MARCO), in addition to lectin-type receptors, detect and bind microbial surface structures, which leads to the uptake of the microbe (185). Microbes coated with serum complement proteins or antibodies are phagocytosed via cell surface complement and Fcγ receptors, respectively (185). Microbe-induced macrophage gene expression is induced primarily via Toll-like receptors (TLRs) and nucleotide-binding

oligomerization domain-like receptors (NLRs). These receptors detect a variety of bacterial and viral structures including lipopolysaccharide (LPS), peptidoglycan (PGN) or viral RNA (2, 188). Macrophages are equipped with a broad set of TLRs and NLRs in order to rapidly detect and respond to a variety of microbes.

Macrophages kill microbes by fusing the microbe-containing phagosomes with lysosomes, which contain proteolytic enzymes. Moreover, activated macrophages produce reactive oxygen and nitrogen intermediates, which further enhance the destruction of the engulfed microbes. Although microbial killing is usually efficient, some intracellular bacteria, including *Listeria monocytogenes*, *Salmonella typhimurium* and *Mycobacterium tuberculosis* are able to disrupt the phagolysosome fusion and persist inside macrophages.

Another key feature of activated macrophages is the production of cytokines and chemokines, which enhances local antimicrobial defence systems. Activated macrophages produce tumor necrosis factor-alpha (TNF-α), interleukin (IL)-6, interferons (IFNs) and a multitude of CC and CXC chemokines. Moreover, depending on the stimulus, several T cell activating cytokines such as IL-12, IL-18, IL-23 and IL-27 are produced (1, 125, 173). Local cytokine environment also modulates the nature and extent of macrophage activation. Classical macrophage activators IFN-γ and TNF-α enhance the production of reactive nitrogen and oxygen intermediates, pro-inflammatory cytokines, and Th1 polarizing cytokines (43, 173). In contrast, IL-4, IL-13 or glucocorticoids reduce the pro-inflammatory response and promote the production of IL-10 and other anti-inflammatory mediators (43, 98, 173). Downregulation of the inflammatory response is important to avoid excess damage to the host and to restore homeostasis after inflammation.

Microbe-induced macrophage activation increases the expression of cell surface MHC and T cell co-stimulatory molecules. This enhances the ability of macrophages to present antigens to T cells. Macrophages are, however, inefficient in priming naïve T cells and their role is more likely to initiate recall responses to previously encountered antigens. (Reviewed in 6, 145)

2.1.2 Dendritic cells

Macrophages and dendritic cells (DCs) share several functions in the antimicrobial defence. Both cell types rapidly recognize microbes, produce cytokines and chemokines and act as APCs. However, DCs are unique APCs in the sense that only DCs are able to activate naïve T cells and thereby initiate *de novo* adaptive immune responses (6, 145). Humans have several specialized DC types including myeloid DCs (mDC), plasmacytoid DCs (pDC) and skin-specific Langerhans cells.

Human mDC and pDCs have both phenotypic and functional differences as they express different PRRs and produce partially distinct set of cytokines. More specifically, mDCs express TLR1, 2, 3, 4, 5, 6, 8, and 10, while pDCs express TLR1, 6, 7, and 9 (63, 74, 92). Thus, pDCs and mDCs respond to different microbes and/or microbial structural components. A specific feature of pDCs is their capacity to produce vast amounts of type I IFNs compared to other cell types (21, 93). In contrast, mDCs produce IL-12 and IL-10, which human pDCs do not produce (93). mDCs and pDCs can be isolated directly from human blood. These circulating DCs are scarce and typically represent only 0,5-2% of peripheral blood mononuclear cells (PBMCs). Thus, most human DC studies have been performed with monocyte-derived DCs (mo-DCs), which can be generated by culturing monocytes in the presence of granulocyte-macrophage colony stimulating factor (GM-CSF) and IL-4 (153). There is some controversy whether human monocytes differentiate into mo-DCs *in vivo*. However, by using an *in vitro* culturing system, monocytes traversing endothelial cell layer have been shown to adopt DC properties (143). The gene expression pattern and functions of mo-DCs resemble those of blood-derived mDCs closely but not completely (72, 96).

DCs patrol in blood and tissues in an immature state and constantly monitor the periphery by capturing antigens via phagocytosis, endocytosis and pinocytosis (6). DCs can also capture microbes by engulfing infected dying cells or by penetrating their dendrites through tight junctions of epithelial cells. A contact with a microbe or another antigen activates a DC maturation process, which results in several drastic changes in DC phenotype and function. During maturation the endocytotic activity of DCs decreases and antigen processing is initiated (6). Moreover, the expression of cell surface MHC and CD80 and CD86 co-stimulatory molecules is enhanced (6). Microbe-activated DCs also produce a variety pro-inflammatory and T cell activating cytokines and chemokines during maturation. Depending on the activation stimulus DCs can produce Th1 polarizing IL-12 family cytokines or tolerance inducing IL-10 (65, 95, 172). It was also recently reported that maturing DCs produce IL-2, which enhances DC-mediated T and NK cell activation (45, 46, 48). One key mechanism rendering DCs so efficient APCs is that mature DCs are able to leave the periphery and home to local lymph nodes, where antigen presentation to T cells takes place. The lymph node homing is mediated by a change in the DC chemokine receptor expression during maturation. Maturing DCs start to express CCR7 and thus DCs become sensitive to CCL19 and CCL21 chemokines which are produced in lymphatic vessels (155, 156).

Although the role of DCs in initiating adaptive immune responses against pathogens has been known for long, it is becoming evident that DCs have a significant role also in maintaining peripheral tolerance and homeostasis. The activation state of DCs

seems to play a significant role in this process. Tolerogenic DCs are generated under steady-state (non-inflammatory) conditions, i.e. when DCs ingest apoptotic cells in the absence of inflammation (172). Tolerogenic DCs have been shown to promote deletion or anergy of autoreactive peripheral T cells which may have escaped the normal thymic selection (central tolerance). A general feature of tolerogenic DCs is that they express T cell co-stimulatory molecules on the cell surface but they do not produce inflammatory cytokines (145, 152, 172).

2.1.3   From innate to adaptive immunity

The function of the innate immunity is to rapidly detect invading microbes and to stop or at least limit the infection. This also gives time for the development of adaptive immunity. Adaptive immunity is antigen-specific and thus offers a more precise and efficient recognition of microbes or their structural components as compared to innate immunity. Most importantly, adaptive immunity generates immunological memory, which enables rapid immune activation upon reinfection. The development of adaptive immunity requires an intimate interplay between APCs and T and B lymphocytes. The main function of B cells is to produce antibodies which protect the host from extracellular microbes and parasites. Upon reinfection memory B cells and circulating serum antibodies rapidly recognize invading pathogens and initiate the activation of immune response. Cytotoxic $CD8^+$ T cells (CTLs) control intracellular pathogens by destroying infected cells via the release of intracellular cytotoxic molecules such as perforin. Both B cells and CTLs require activating signals from $CD4^+$ T helper (Th) cells. The polarization of $CD4^+$ Th cells determines whether adaptive immune response is Th1 type (CTLs) or Th2 type (B cells). This classical Th1/Th2 paradigm has been recently updated after additional effector T cell types have been identified. IL-17 producing Th17 cells play a central role in the pathology of inflammatory autoimmune diseases (79). These cells may also mediate host response against certain pathogens (54, 79). Moreover, at least three regulatory T cell types have been characterized. Regulatory T cells are important in maintaining peripheral tolerance and limiting extensive immune response via direct cell-cell contacts and/or by the production of suppressive cytokines IL-10 and transforming growth factor (TGF)-β (116, 172).

The activation and polarization of naïve Th0 cells requires three signals from APCs. The first signal consists of processed microbe-derived peptides, which are presented to T cells in the context of APC MHC molecules. The peptide-MHC complex is recognized by cognate T cell receptor (TCR) on T cell surface. The second signal is delivered by co-stimulatory molecules such as CD80 and CD86 which are recognized by T cell CD28 receptor. Co-stimulation is important as it activates

intracellular signalling in both APCs and T cells. The third signal determines the polarization of Th0 cells. Soluble APC-derived cytokines and several cell surface receptors transfer the polarization signals to T cells. The classical Th1 polarization factors include type I and II IFNs, IL-12, IL-18 and IL-27 (65, 177). Th17 polarization is mediated by TGF-β, IL-6 and IL-23 (65, 79, 195). The mediators of Th2 polarization are still somewhat ill defined but the lack of Th1 polarizing cytokines, expression of IL-4, prostaglandin E2 (PGE2), or histamine and cell surface OX40-OX40L receptor pair plays a role here (27, 50, 76). Cytokine-mediated signalling in antigen presentation is not unidirectional. For example T cell-derived IFN-γ enhances APC activation while IL-10, IL-21 or TGF-β can downmodulate APC functions and promote homeostasis. To sum up, a complex network involving both cell-cell contacts and soluble mediators determines the development of adaptive immune response.

2.2   Microbe recognition by the innate immune system

In order to discriminate harmful pathogens from "self" the immune system utilizes PRRs. PRRs detect conserved structures that are found in microbes but are absent from the host (71). Such structures are called pathogen associated microbial patterns (PAMPs) and they include i.e. bacterial lipopolysaccharide (LPS), peptidoglycan (PGN) and unmethylated CpG-rich DNA (2, 188). A general property of PAMPs is that they are structurally versatile and include lipids, proteins, carbohydrates and nucleic acids. PRRs can be classified into families based on the domains the receptors have. The most common are lectin, cysteine-rich and leucine-rich repeat domains (107). The main functions of PRRs include the activation of complement cascade, enhancement of phagocytosis and initiation of intracellular signalling and gene expression.

In a broad sense many serum proteins including certain complement proteins, mannan binding lectin (MBL), C-reactive protein (CRP) and serum amyloid P-protein (SAP) can be classified as PRRs. These proteins bind to microbial surface proteins, carbohydrates or lipid structures. Serum factors initiate complement-mediated microbial lysis and promote phagocytosis of microbes. Reviewed in (71, 107)

Professional immune cells express a variety of PRRs on their cell surfaces and intracellular compartments. Scavenger receptor family includes several structurally unrelated cell surface receptors that mediate phagocytosis of bacteria. For example, type I and II scavenger receptor A (SR-AI/II) binds LPS and lipoteichoic acid (LTA) and enhances the uptake of *Escherichia coli* and *Staphylococcus aureus* by macrophages (30, 52, 139). *In vivo* experiments performed with SR-AI/II knockout

mice have demonstrated that these mice are more susceptible to *S. aureus* infection compared to wild type mice (186). Other scavenger receptors, such as MARCO, CD36 and lectin-like oxidized low density lipoprotein receptor (LOX)-1 bind both Gram-negative and Gram-positive bacteria (32, 33, 163, 175). Another family of phagocytosis receptors is C-type lectins. Several C-type lectin receptors have been cloned but the specific microbial ligands have remained largely uncharacterized. Most information is available on macrophage mannose receptor (MMR), which detects mannose and fucose residues on microbial surfaces (185). The DC-restricted receptor dendritic-cell-specific ICAM3-grabbing non-integrin (DC-SIGN) binds mannan, mannosyl-lipoarabinomannan and modified Lewis blood group antigens and this receptor has been shown to mediate the uptake of mycobacteria and HIV by DCs (13). Dectin-1 receptor is expressed on macrophages and DCs and this receptor detects β-glucan structures found in yeast cell wall (13, 185).

The above discussed PRRs have traditionally been thought to mediate microbial detection and phagocytosis but not the activation of gene expression. The discovery of TLRs and later on NLRs and caspase recruitment domain (CARD)-helicase receptors has integrated microbial recognition to the activation of intracellular signal transduction and gene expression. The distinction between signalling and phagocytosis receptors is, however, likely to change as recent reports have demonstrated that engagement of Dectin-1 and DC-SIGN activates intracellular signalling and cytokine production (14, 148, 189).

2.2.1   Toll-like receptors

The involvement of TLRs in innate immunity was first demonstrated in Drosophila where TLRs were found to be essential for anti-fungal and anti-bacterial defence (88, 197). Subsequently, TLR homologs were described in humans (108, 146). Currently 12 mammalian TLRs have been identified (2, 188). TLRs are most prominently expressed by professional antigen presenting cells including macrophages and dendritic cells. In contrast, TLRs are usually expressed at low or undetectable levels by epithelial or endothelial cells. Inflammatory signals, such as cytokines or microbial attack can, however, enhance TLR expression also in non-immune cells (2). TLRs are germ line-encoded type I integral membrane glycoproteins. The extracellular domain of TLRs contains a leucine-rich repeat (LRR) motif, which mediates PAMP detection. The cytoplasmic signalling domain of TLRs is homologous to interleukin 1 receptor and thus named Toll/IL-1R (TIR) domain. The intracellular domain is essential for the activation of intracellular signalling.

The first TLR ligand described was bacterial LPS, which is detected by TLR4 (142). In recent years the ligands for several but not all TLRs have been identified (summarized in Table 1). Significant variability in the structures of TLR ligands exists and they can consist of lipids, proteins or nucleic acids. Interestingly, some TLRs can detect ligands from several of these categories (2). Further fine-tuning on the ligand specificity is achieved by formation of TLR heterodimers. For example the TLR1/TLR2 dimer detects bacterial triacyl lipopeptides, while TLR2/TLR6 recognizes diacyl lipopeptide structures (183). At least some TLRs require accessory molecules for PAMP detection. For example TLR4 requires MD2, CD14 and LPS-binding protein (LBP) for efficient signal transduction, while CD36 mediates the detection of diacylglyserols by TLR2/TLR6 dimer (59, 73, 142, 164). The cellular location of TLRs varies. TLRs 1, 2, 4, 5 and 6 are expressed on the cell surface while TLRs 3, 7, 8 and 9 are found intracellularly in endosomes (2, 182). Intracellular TLRs detect nucleic acids which are also produced by the host. Thus, it is likely that the endosomal location of these TLRs prevents uncontrolled immune activation by endogenous nucleic acids.

TLR signalling is initiated by ligand binding, which results in the dimerization or multimerization of TLRs. Subsequently, TIR-domain-containing adaptor molecules are recruited to the intracellular domain of TLRs. Five adaptor molecules have been identified. These include myeloid differentiation factor 88 (MyD88), TIR-domain-containing adaptor inducing IFN-β (TRIF), TIR-domain containing adaptor protein (TIRAP), TRIF-related adaptor molecule (TRAM) and Sterile alpha and HEAT/Armadillo motif protein (SARM). The key players here are MyD88 and TRIF, which mediate the activation of downstream signalling (2). MyD88 is used by all TLRs except TLR3, while TRIF signalling is used by TLR3 and TLR4. TIRAP/MAL is required for interaction of MyD88 with TLR2 and TLR4, while TRAM enables TLR4-TRIF interaction (2, 134). The fifth adaptor protein SARM was recently shown to negatively regulate TRIF signalling (15). Downstream TLR signalling includes several phosphorylation and ubiquitination steps where TNF receptor associated factor (TRAF) proteins and interleukin-1 receptor associated kinases (IRAKs) together with cellular ubiquitination machinery play a key role (2, 19). Ultimately, TLR signalling results in the activation and nuclear translocation of nuclear factor kappaB (NF-κB), interferon regulatory factors (IRFs) and mitogen-activated protein kinase (MAPK)-regulated transcription factors. These transcription factors regulate the expression of a vast majority of anti-microbial genes including pro-inflammatory cytokines, chemokines and IFNs.

Table 1. Microbial ligands of human TLRs

Receptor	Ligand	Origin of ligand
TLR1+TLR2	Triacyl lipopeptides	Bacteria and mycobacteria
TLR2	(Peptidoglycan)	Gram-positive bacteria
	Porins	*Neisseria*
	Lipoarabinomannan	*Mycobacteria*
	Phospholipomannan	*Candida albicans*
	Glucuronoxylomannan	*Cryptococcus neoformans*
	Hemagglutin protein	Measles virus
	Not known	HSV1, HMCV
	tGPI-mutin	*Trypanosoma*
TLR3	dsRNA	RNA viruses
TLR4	LPS	Gram-negative bacteria
	Mannan	*Candida albicans*
	Glucuronoxylomannan	*Cryptococcus neoformans*
	Envelope proteins	RSV
	HSP60, 70	Host
	Fibrinogen	Host
TLR5	Flagellin	Flagellated bacteria
TLR2+TLR6	Lipoteichoic acid	Group B *Streptococcus*
	Diacyl lipopeptides	Mycoplasma
	Zymosan	*Saccharomyces cerevisiae*
TLR7/8	ssRNA	Viruses
TLR9	CpG-DNA	Bacteria, mycobacteria and viruses

Adapted from (2, 188)

2.2.2   Cytoplasmic sensors

In addition to TLRs, two cytoplasmic PRR families have been recently described, namely nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and caspase-recruitment domain (CARD)-helicase proteins.

NLRs are expressed in APCs and epithelial cells. It has been suggested that NLRs may be especially important in intestinal epithelial cells which are mostly devoid of TLRs in order to prevent continuous inflammation. Moreover, NLRs are likely to play a role in the detection of certain intracellular bacteria that may escape from the phagosomes to the cytoplasm. Two key functions for NLRs have been demonstrated. Some NLRs mediate the activation of intracellular signalling pathways, while others activate caspase proteases. Reviewed in (24, 103).

Similar to TLRs, NLRs contain a C-terminal LRR-domain, which is involved in ligand sensing. The N-terminal part of NLRs mediates protein-protein interactions and signalling. The N-terminus can contain a pyrin domain (PYD), CARD, or baculovirus inhibitor of apoptosis repeat (BIR) domain (24, 103). Over 20 NLRs have been identified in the human genome but the function or the specific ligand has been identified only for few of them. Most information is available on NOD1 and NOD2 receptors. NOD1 detects meso-diaminopimelic acid which is a part of Gram-negative peptidoglycan (17, 40). The ligand for NOD2 is muramyl dipeptide found in both Gram-negative and Gram-positive bacteria (41, 68). NOD1 and NOD2 are the only NLRs that have thus far been shown to induce intracellular signalling. These receptors activate NF-κB and MAPK signalling in receptor-interacting protein (RIP)2-dependent fashion (67, 81). In monocytes and DCs NOD1/2 ligands alone are relatively weak activators of pro-inflammatory responses but they can act in synergy with TLR ligands thus enhancing the inflammatory cytokine response (37, 179). NOD2 ligands have, however, also been shown to reduce TLR-induced IL-12 production (193, 194) suggesting that the interactions between TLR and NLR pathways may vary considerably depending on cell type, activating stimulus or the target gene. Interestingly, frameshift mutations in NOD2 gene are associated with increased incidence of Crohn´s disease (102). The mechanisms of how defects in NOD2 protein result in the ongoing intestinal inflammation are still uncertain but the enhanced production of IL-12 and resulting Th1 polarization may play a key role (194). Moreover, defective NOD2-proteins have been shown to reduce the production of intestinal antimicrobial peptides which probably weakens the clearance of pathogenic microbes and may render host more susceptible to intestinal inflammation (81).

Another key feature of certain NLRs is their ability to activate caspases, which are crucial for the posttranslational processing and activation of IL-1β and IL-18

cytokines. Caspases also mediate cellular apoptosis. Microbe-induced NLR activation may therefore play a key role in the production certain inflammatory mediators and, on the other hand, regulate controlled cell death during the microbial attack. Thus far the ability to activate caspase-1 has been shown for two NLRs, namely Cryopyrin and ICE protease-activating factor (Ipaf) (36). Cryopyrin is activated by bacterial RNA, certain antiviral compounds, toxins and endogenous uric acid crystals while Ipaf detects bacterial flagellin (75, 99, 101, 110, 176).

The most recently identified PRR family is the CARD-helicase family which has three members; retinoid acid inducible gene-I (RIG-I), melanoma differentiation associated gene-5 (mda5) and LGP2 (3, 151, 202). CARD-helicases are intracellular sensors of viral dsRNA. The helicase domain of these receptors binds dsRNA while the N-terminal CARD domain is responsible for protein-protein interactions and signalling. LGP2 lacks the N-terminal CARD domain and this protein has been suggested to be a negative regulator of RIG-I and mda5 (151). Activated CARD-helicases interact with IFN-β promoter-stimulating protein (IPS)-1 adaptor protein. The following downstream signalling cascade converges with the TLR signalling and results in the activation of NF-κB and IRF transcription factors.
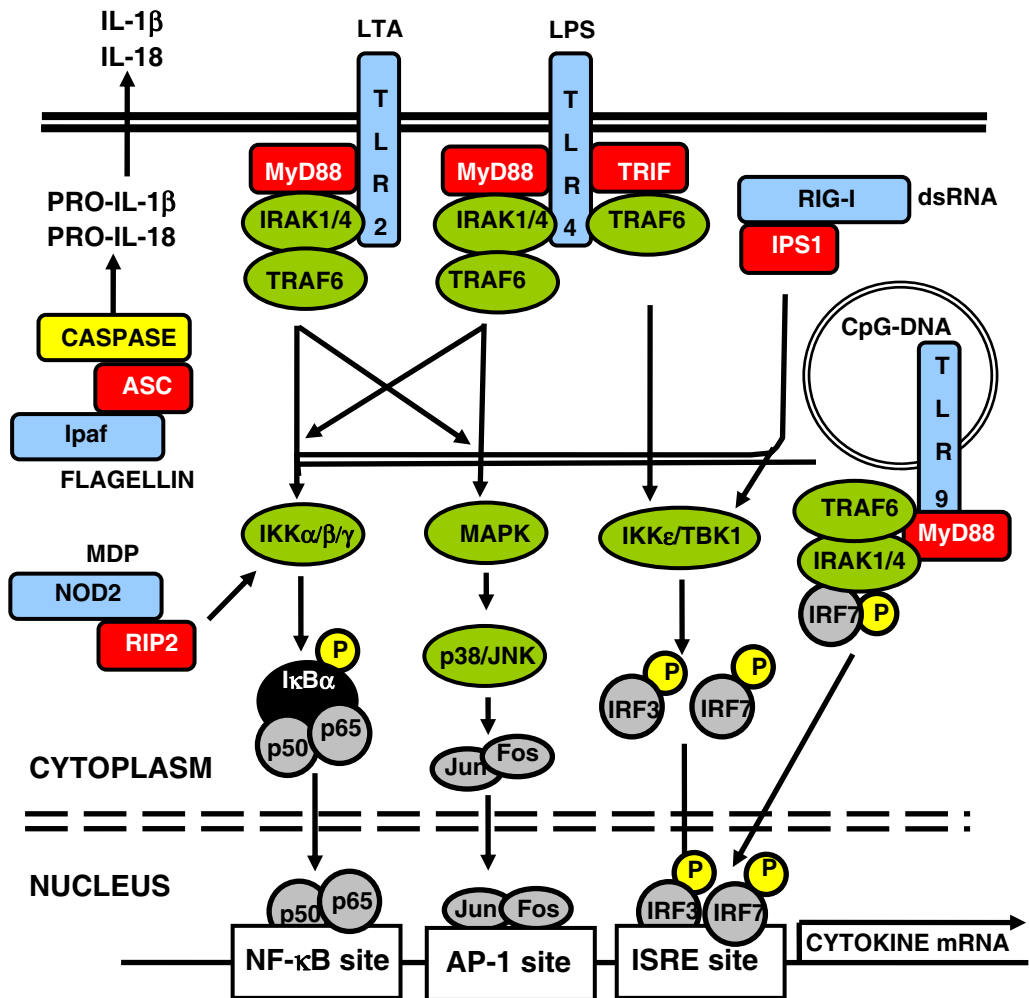
Figure 1. *Schematic view of microbe detection and signal transduction via pattern recognition receptors (PRRs). Toll-like receptors (TLRs) can be expressed on cell surface or in intracellular endosomes. Ligand binding to TLR recruits adaptor molecules and initiates multistep signal transduction, which involves phosphorylation and/or ubiquitination of transcription factors. Depending on TLR in question, NF-κB, MAPK and/or IRF signal transduction pathways can be activated. Cytoplasmic microbial sensors include nucleotide-binding oligomerization domain like receptors (NLRs) and caspase-recruitment domain (CARD)-helicases. Certain NLRs, such as NOD2, induce NF-κB activation while Ipaf mediates caspase-1 activation and proteolytic processing of cytokines instead. CARD-helicases, including RIG-I, detect viral dsRNA and activate NF-κB and IRF signal transduction pathways. Receptors are coloured blue and adaptor proteins in red. Signal transduction components are marked green and transcription factors grey. Ligands for each receptor are indicated.*

2.3   Cytokines

In multicellular organisms effective means to control the interactions between different cells are required. Cytokines are soluble polypeptides which act as messengers between cells and contribute to nearly all steps of the immune response. Cytokines regulate the proliferation, differentiation, and recruitment of cells to appropriate location during homeostasis and inflammation. Cytokines regulate target cell functions by binding to their specific cell surface receptors, which initiate intracellular signalling pathways and gene expression. A general feature of cytokines is their pleitoropic and redundant effects. Thus, several cytokines may have similar or overlapping functions and compensate each others functions. Moreover, different cytokines can also act together and synergistically modulate target cell functions. Cytokines are produced locally which distinguishes them from hormones, which have more systemic effects. Cytokines are grouped into families based on their structural and/or functional similarities. However, for some cytokines the structural relationship is less evident and the classification is based mainly on proposed tertiary structure and spatial organisation. Cytokine families are summarized in Table 2.

Table 2. Cytokine families

Cytokine family	Members	Biological activities
Interleukins	IL-1 - IL-33	Regulation of cell proliferation and differentiation, immune cell activation/repression
Interferons	IFN-$\alpha,\beta,\gamma,\lambda,\omega,\tau$	Antiviral, anti-proliferative, immunomodulation
Tumor necrosis factors	TNF-α, TNF-β	Proinflammatory, apoptosis
Transforming growth factor-β	TGF-β, inhibins, activins, bone morphogenetic proteins	Immunomodulation, development, morphogenesis
Chemokines	C, CC, CXC, CX3C type chemokines	Leukocyte chemotaxis, angiogenesis/ angiostasis, tumor metastasis, HIV infection, polarization of adaptive immune response
Colony stimulating factors	GM-CSF, G-CSF, M-CSF, IL-3	Growth and differentiation of hematopoietic cells
Growth factors	EPO, TPO, EGF, FGF, PDGF	Growth and differentiation of cells

Adapted from (196)

2.3.1 Inflammatory cytokines

Inflammatory cytokines are produced at the first wave of microbe-induced stress. Common to these cytokines is that they are produced by nearly all cell types and that they promote the activation of anti-microbial immune defence systems. The classical pro-inflammatory cytokines include TNF-α, IL-1α/β, IL-6 and Type I IFNs. These cytokines act on leukocytes as well as on cells of non-immune origin. Professional immune cells, especially the cells of monocyte/macrophage/DC lineage also produce cytokines able to activate adaptive immune response. The key cytokines regulating Th1 polarization are IFNs, IL-12 and IL-27 (65, 177). In contrast, IL-10 and TGF-β downregulate inflammatory response and enhance the generation of regulatory T cells (49, 116). The major characteristics of the inflammatory cytokines analyzed in this thesis work are described below.

Tumor necrosis factor-α

TNF-α is a multifunctional pro-inflammatory cytokine. Environmental stress, such as bacterial or virus infection, components of complement cascade, oxygen radicals or radiation can induce TNF-α production (192). TNF-α is produced in high levels by professional immune cells but it is also inducible in non-immune cells such as epithelial cells and fibroblasts. TNF-α is a very potent immune activator and its production is tightly controlled. TNF-α gene expression is mediated by p38 and c-jun NH2-terminal kinase (JNK) MAP kinases, which activate the downstream signalling molecules activating transcription factor (ATF)-1, ATF-2, ETS-like kinase protein (Elk)-1, c-jun and possibly also NF-κB (192, 204). TNF-α production is also regulated post-translationally by the cleavage of pro-TNF-α polypeptide into its mature form. Binding of TNF-α to its cell surface receptor activates MAPK and NF-κB transcription factors, which regulate the gene expression of several PRRs, transcription factors, cytokines and anti-microbial mediators (192). Additionally, the intracellular death domain of TNF-α receptor may trigger caspase activation and apoptosis upon TNF-α binding (86). Uncontrolled production of TNF-α is involved in several human inflammatory diseases such as Crohn´s disease, arthritis and sepsis.

Interferons

Interferons were initially described as agents that are able to inhibit virus replication. Later on several other important immunomodulatory effects for IFNs have been described. Interferons can be categorized into three classes. Type I IFNs include IFN-β and 13 extremely homologous IFN-α subtypes. Type I IFNs are produced by

nearly all cells but APCs, especially pDCs, produce the highest levels of these cytokines (93, 171). Type I IFNs regulate the expression of several genes involved in the innate immune response. The expression of many PRRs, transcription factors, cytokines, chemokines and cytokine receptors is enhanced by IFNs (83, 171). Moreover, type I IFNs induce the production of important antiviral proteins including MxA, (2´-5´)oligo(A) synthetase (OAS) and protein kinase R (PKR) (83, 171). Type I IFNs also regulate the development of adaptive immunity. They enhance antigen processing, DC maturation and promote Th1 polarization (10, 83). Type I IFNs modulate gene expression by activating the Janus kinase-signal transducer and activator of transcription (Jak-STAT) transcription factor pathway (90).

IFN-γ is the only type II IFN. This cytokine is primarily produced by T and NK cells although there is evidence that APCs can also produce IFN-γ in response to an appropriate stimulus. IFN-γ has an important role in dictating T cell polarization as it promotes Th1 and inhibits Th2 polarization of naïve T cells (177). IFN-γ also enhances macrophage activation and cytokine production and several chemokines, including CCL2, CCL3, CCL5, CXCL9 and CXCL10 are IFN-γ regulated. IFN-γ also enhances NK cell activation (43, 160).

The most recently discovered IFN class is type III IFNs, also known as IFN-λs, which consist of three homologous genes IFN-λ1-3 (also designated IL-28A, IL-28B and IL-29) (82, 162). Type I and III IFNs have similar antiviral functions but the genes are located in different chromosomes and they use different cell surface receptors to mediate their functions (82, 106, 137, 162).

IL-12 cytokine family

 The members of the IL-12 cytokine family, namely IL-12, IL-23 and IL-27, regulate T cell activation and polarization. These cytokines are produced primarily by macrophages and dendritic cells. IL-12 family cytokines are heterodimers. IL-12 is composed of p35 and p40 subunits, IL-23 is made of p19 and p40 subunits, and IL-27 is composed of p28 and EBI3 (65). APC-derived IL-12 has long been known to be crucial for the development of Th1 cells and to induce T cell IFN-γ production (65, 177). IL-23 and IL-27 were also initially described as Th1 cytokines but recently their role in the regulation of T cell response has been clarified. IL-23 has been shown to promote the expansion of the newly discovered Th17 T cells, which contribute to several autoimmune inflammatory conditions (54, 79). IL-27 promotes Th1 polarization and suppresses the development of Th17 cells (23, 65). IL-12 family of cytokines mediate their actions through the Jak-STAT signal transduction pathway (90).

2.3.2 Chemokines

One key element of a properly functioning immune system is the coordinated movement of immune cells. Depending on the type of microbe or site of inflammation right kinds of immune cells have to be recruited. Chemokines or **chemo**tactic cyto**kines** are responsible for controlling the leukocyte movement. Nearly all cell types are able to produce these proteins in response to microbes or cytokine stimulus. In recent years several functions, in addition to chemotaxis, have been described for chemokines. Chemokines and chemokine receptors have been shown to be involved in embryogenesis, organ development, angiogenesis/ angiostasis, tumor metastasis, and binding of HI-virus to its target cells. Currently over 50 human chemokines have been described. Reviewed in (85, 135, 150, 205)

Chemokines are small 8- to 15-kD proteins and they can be classified into four families on the basis of conserved N-terminal cysteine residues. In the CC family chemokines the two cysteines are adjacent, while in the CXC family chemokines there is one intervening amino acid between the cysteine residues. Most known chemokines belong to these CC or CXC families. The two minor chemokine groups are CX3C and C families which have only one and two members, respectively. The CXC chemokines can be further divided into ELR (Glu-Leu-Arg)-motif positive and –negative chemokines. This has also a functional relevance, since ELR$^+$CXC chemokines act as angiogenic factors, while most ELR$^-$CXC chemokines are angiostatic (119, 174). Although different chemokines have overlapping functions in leukocyte chemotaxis some generalizations on their target cells can be made. The CC family chemokines are chemoattractants to one or several types of mononuclear leukocytes, eosinophils or basophils (85, 94). In contrast, ELR$^+$CXC chemokines attract neutrophils and ELR$^-$CXC chemokines recruit T lymphocytes (85, 94). Chemokines can also be classified on the basis of their functionality into homeostatic and inflammatory chemokines. Members of the former subset regulate leukocyte haematopoiesis in the bone marrow and thymus, and guide the leukocyte migration to spleen and lymph nodes. Inflammatory chemokines primarily control leukocyte traffic during an acute infection, inflammation or tissue injury. Inflammatory chemokines include several members of the CC and CXC chemokines and they mainly attract monocytes, neutrophils, NK cells, immature DCs and T cells. These cells have a key role in innate and adaptive anti-microbial defence. Some inflammatory chemokines, including CCL2 and CCL5, have also been shown to take part in T cell polarization (135, 199). In this thesis work the focus has been in analysing the production and gene regulation of inflammatory chemokines during microbial attack. The properties of inflammatory chemokines analyzed in this study are summarized in Table 3.

Inflammatory chemokines are produced locally in infected tissues where they form a chemokine gradient by binding to extracellular matrix (150, 154). The gradient is recognized by leukocytes via their cell surface chemokine receptors. Binding of a chemokine to its receptor results in the expression of adhesion molecules, tissue modifying enzymes and leads to rearrangement of the cytoskeleton. These functional changes enable rolling leukocytes to bind endothelium and to transmigrate through cell junctions to the site of inflammation to perform their effector functions.

Chemokines transduce signals via G-protein coupled, seven-transmembrane domain receptors, also known as serpentine receptors. The receptors are also categorized into CCR, CXCR, CX3CR and CR families. Currently 19 human chemokine receptors are known (150, 205). The key element of chemokine system is that the expression of chemokine receptor determines cell type specificity of a given chemokine. There is, however, significant redundancy in the chemokine system, since some chemokine receptors can bind multiple ligands with different affinity. Therefore, several chemokines have overlapping functions and can compensate each other. Ligand binding to chemokine receptor results in receptor dimerization and uptake of receptor to intracellular compartments. Receptor uptake desensitizes cells for further chemokine stimulus. Activated receptor is bound to G proteins and initiates multiple downstream signalling cascades including phospholipase C (PLC), phosphoinositode-3 kinase-gamma (PI3Kγ), and MAP kinases (109). Some chemokines, including CCL5 and CXCL12, also activate the Jak-STAT pathway (144, 147).

Chemokine production and the expression of chemokine receptors is not static but may change during the course of inflammation. One example of this is seen during microbe-induced DC maturation. Immature DCs are recruited to infected tissues via CCR1, 2, 3, 4, and especially CCR6 binding chemokines (51). Maturation leads to a change in chemokine receptor pattern as CCR6 is down-regulated and the lymph node-homing receptor CCR7 is up-regulated (156). Mature DCs thus respond to CCR7 ligands CCL19 and CCL21, which are produced in lymphatic vessels, and are directed to local lymph nodes, where antigen presentation to T cells may take place (51, 169).

Table 3. Inflammatory chemokines analysed in the present study

Chemokine	Additional name(s)	Receptor(s)	Target cell(s)
CCL2	MCP-1, MCAF	CCR2,10	Monocytes, basophils, T and NK cells
CCL3	MIP-1α	CCR1,5	Monocytes, granulocytes, T and NK cells, DCs
CCL5	RANTES	CCR1,3,5	Monocytes, granulocytes, T and NK cells, DCs
CCL7	MCP-3	CCR1,2,3,10	Monocytes, granulocytes, T and NK cells, DCs
CCL19	MIP-3β, ELC, Exodus-3	CCR7	Mature DCs, T cells, B cells, activated NK cells
CCL20	MIP-3α, LARC, Exodus-1	CCR6	Immature DCs, T cells
CXCL8	IL-8	CXCR1,2	Neutrophils, T cells
CXCL9	MIG	CXCR3	NK and Th1 cells
CXCL10	IP-10	CXCR3	NK and Th1 cells

Adapted from (22, 85, 150)

2.3.3 Regulation of cytokine and chemokine gene expression

Inflammatory cytokines and chemokines are produced rapidly following microbial attack. The expression of these genes is controlled primarily at a transcriptional level by binding of transcription factors to their target elements on gene promoters. Most cytokine and chemokine genes are regulated by NF-κB, IRF, STAT and MAPK-activated transcription factors. These are also the transcription factor pathways most prominently activated via pattern recognition receptors. However, cytokines themselves also activate these transcription factors, thereby creating positive (and negative) feedback mechanisms. Thus, both direct (PRR-mediated) and indirect (cytokine-mediated) mechanisms are involved in the regulation of cytokine and chemokine production during microbial infections.

Nuclear factor kappa-B pathway

The NF-κB pathway is essential in controlling innate and adaptive immune responses. During the activation of innate immunity NF-κB regulates the expression of several cytokines, chemokines, antimicrobial peptides and stress response proteins (91, 180). NF-κB is a dimeric transcription factor which consists of five members, namely p65 (RelA), p50, p52, c-Rel and RelB. Under steady-state conditions NF-κB proteins are retained in the cytoplasm in association with inhibitory-κB (IκB) proteins. Upon activation, the inhibitory proteins are phosphorylated by IκB kinases (IKKα/β/γ) and subsequently targeted for degradation, which enables the translocation of NF-κB dimers into nucleus (91). In the nucleus NK-κB dimers bind to their target DNA sequences and associate with co-activation proteins such as cAMP responsive element binding (CREB) protein to enhance transcription. NF-κB pathway is induced by a variety of signals including cytokines, PRR-mediated microbe detection or DNA damage (91, 180).

NF-κB target gene specificity is obtained by several mechanisms. Different NF-κB proteins may form heterodimers with distinct DNA sequence preference. Also the expression of NF-κB components varies in different cell types. Recent evidence suggests that the efficiency of NF-κB dimer formation, DNA binding and association with co-activator proteins can be controlled by phosphorylation, acetylation or ubiquitination of NF-κB components. Reviewed in (19, 201).

Interferon regulatory factors (IRFs)

The IRF family of transcription factors consists of nine members. In recent years it has become evident that IRF activation via TLRs and other PRRs has a critical role

in modulating the activation of innate immune response. IRF proteins contain a conserved DNA binding motif which binds to interferon stimulation responsive element (ISRE) sequences on target gene promoters. The ISREs were initially described in the promoters of IFN genes but they are also found in several other genes controlling immune defence and cellular differentiation (61, 184). Although a consensus ISRE sequence has been established it is likely that different IRF homo- or heterodimers have unique sequence preference, which creates specificity on their target genes. IRFs can also interact with other transcription factors and transcriptional regulators thereby generating further selectivity on target sequences. In the context of cytokine and chemokine expression IRF1, IRF3, IRF4, IRF5 IRF7, IRF8 and IRF9 have been most extensively studied. IRF1 controls the expression of several innate immune genes including NOS2, gp91^{PHOX}, IL-12, IL-15 and caspase-1 (61, 184). The expression of type I IFNs and some chemokines is controlled by IRF3 and IRF7, which are retained in the cytoplasm under steady-state conditions. Upon TLR triggering IRF3 and IRF7 are serine phoshorylated by TANK-binding kinase (TBK)-1 and IKKε kinases, which enables nuclear translocation and DNA binding of IRF3/7 (35, 161). The induction of IFNs and involvement of IRF3/7 in this process is, however, also cell type and TLR-specific since in pDCs IRF7 interacts directly with TLR7/9-MyD88-TRAF6 complex and induces rapid and efficient IFN production (60). TLR-induced expression of pro-inflammatory cytokines TNF-α, IL-6 and IL-12 was recently shown to be mediated by IRF5 (181). Upon TLR-activation IRF5 interacts directly with TLR-MyD88-TRAF6 complex which leads to IRF5 nuclear localization and gene expression. It was also demonstrated that IRF5-mediated cytokine response is negatively regulated by IRF4 (62, 127). IRF8 regulates microbe-induced IL-12 production and the development of certain (mouse) DC types (39, 158, 159). IRF9 is constantly localized in the nucleus and it associates with STAT1 and STAT2 upon IFN-α/β stimulation. This complex is termed interferon stimulated gene factor (ISGF)3 and it regulates the expression of IFN-inducible genes (53, 80).

Signal transducers and activators of transcription (STATs)

STAT transcription factor family has seven members; STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6. STATs mediate intracellular signalling of several cytokines, growth factors, hormones and chemokines. STAT activation is initiated by binding of ligand to its cell surface receptor. Ligand binding results in receptor multimerization and phosphorylation of receptor-associated Jaks. This leads to recruitment and subsequent phosphorylation of STATs, which form dimers or multimers and localize into nucleus. STATs bind to gamma-activated site (GAS) or ISRE elements on target gene promoters and gene specificity is achieved by distinct

sequence preference of different STAT dimers. Four Jaks have been described and they associate with different cytokine receptors, which creates specificity to cytokine-induced STAT-mediated gene expression. Reviewed in (89, 90).

STATs regulate the expression of several important immune response genes. These include many chemokines, co-stimulatory molecules, MHC class I and II molecules, and complement proteins (89, 90, 171). These genes are often activated by IFN-induced STAT1 and/or STAT2 activation. Type I IFNs induce the formation of STAT1/2 heterodimers, which can associate with IRF9. This ISGF3 complex binds to ISRE/GAS sites on several cytokine and chemokine genes. IFN-γ, in contrast, induces STAT1 homodimerization which in part explains the differences in target genes of type I and type II IFNs (160). STATs also regulate cellular differentiation and the development of adaptive immunity. T cell polarization is critically regulated by STAT4 and STAT6. Type I IFNs and IL-12 activate STAT4 which results in Th1 polarization, while IL-4 and IL-13 induce STAT6 activation and drive Th2 polarization (200).

2.4   Gram-positive bacteria

Eubacteria can be classified into Gram-negative and Gram-positive bacteria on the basis of bacterial cell wall structure. The cell wall of Gram-negative bacteria is multilayered and consists of LPS on the outer cell membrane and smaller amounts of PGN. In contrast, the cell wall of Gram-positive bacteria is composed of a single layer of PGN together with teichoic and lipoteichoic acids. In this thesis work the activation of macrophage and dendritic cell inflammatory cytokine and chemokine response to Gram-positive bacteria was analyzed. Pathogenic *Streptococcus pyogenes* and non-pathogenic *Lactobacillus rhamnosus* were used as model bacteria.

2.4.1   Streptococcus pyogenes

S. pyogenes (Group A streptococcus) is an important pathogen, which exclusively infects humans. The infections caused by *S. pyogenes* vary from relatively mild and self-limiting skin and throat infections to severe tissue damage (115). However, humans are also often non-symptomatic carriers of this bacterium. *S. pyogenes* has several virulence factors. The invasion and spreading of *S. pyogenes* is promoted by the production of extracellular proteins including steptolysin S and O, several proteases and streptococcal DNAse (26). The M protein and hyaluronic acid capsule inhibit opsonin-mediated phagocytosis of *S. pyogenes* (26, 31). Moreover, *S. pyogenes* produces at least seven exotoxins which act as superantigens and induce non-specific activation and cytokine production of T cells (26, 105, 170). The innate

immune defence against *S. pyogenes* relies on neutrophils and macrophages, which phagocytose and kill *S. pyogenes*. A critical role of macrophages in controlling the spread of *S. pyogenes* and systemic bacterial load was recently demonstrated in mice depleted of macrophages. Macrophage-depleted mice showed high mortality in response to the infection, while control mice survived the bacterial challenge (42). The ability of *S. pyogenes* or its LTA and exotoxins to induce inflammatory cytokine production in whole blood, PBMCs, and monocytes has been well documented (56, 122, 123, 129, 190). However, *S. pyogenes*–induced macrophage and dendritic cell activation is relatively poorly characterized. The PRRs mediating *S. pyogenes*-induced gene expression have not been analyzed in detail. Data from our laboratory indicate that TLR2, the stereotypical receptor for Gram-positive bacteria, at least partially mediates *S. pyogenes*-induced NF-κB activation in macrophages (Miettinen et al unpublished data). Data from other streptococci indicate that both TLR-dependent and TLR-independent mechanisms may be involved in streptococci-induced intracellular signalling. For example, the expression of several inflammatory cytokine and chemokine genes was delayed but not absent in Group B streptococci-stimulated TLR2 knockout macrophages (29, 57, 58). The contribution of intracellular NOD2 receptor has been demonstrated in pneumococci-induced NF-κB activation (136). Gram-positive bacteria-induced signal transduction can also be induced via scavenger receptors. For example CD36 mediates the uptake and intracellular signalling of *S. aureus* (175). Data from experiments performed with other bacteria should, however, be generalized with care since even subtle differences in cell wall protein composition and structure, or the expression of protective capsule can significantly alter the uptake, detection and intracellular signalling induced by different bacterial species. Moreover, the receptor usage is likely to vary between different cell types.

2.4.2 *Lactobacillus rhamnosus*

L. rhamnosus is a non-pathogenic lactic acid bacterium originally isolated from the human intestine. *L. rhamnosus* is widely used in dairy industry as a probiotic supplement. In recent years increasing interest has focused on probiotic bacteria. Most probiotic bacteria used and studied belong to *Lactobacillus* or *Bifidobacterium* genera. The best documented beneficial effects of probiotic bacteria include reduced susceptibility to diarrhea caused by rotavirus infection or antibiotic treatment (25, 178, 191). In addition, probiotic bacteria have been shown to have immunomodulatory effects including decreased incidence of atopy or allergy and reduction of the symptoms of chronic intestinal inflammation (reviewed in 157). The clinical data is still preliminary and partially contradictory and additional well controlled studies are required to elucidate the potential of probiotics in treating

these diseases. Moreover, the mechanisms are ill defined and rather speculative. It has been suggested that probiotic bacteria-induced cytokine production or modulation of intestinal APC functions by these bacteria could balance and/or enhance the functionality of intestinal immune system.

Probiotic bacteria-induced cytokine production has been studied in several models. Human PBMCs produce both pro-inflammatory and immunosuppressive cytokines, including IL-10 and TGF-β in response to *Lactobacillus* species (18, 112, 114, 133). In these studies the differences between different bacterial species have been mostly quantitative. Dendritic cells are an important link between innate and adaptive immune responses and, depending on the stimulus, DCs can induce Th1, Th2 or tolerogenic T cell response. Thus, the modulation of DC functions could provide one mechanism for the proposed immunological effects of probiotic bacteria. Several recent reports have addressed the ability of different *Lactobacilli* to induce cytokine production in DCs. In contrast to PBMCs, DCs show dramatic bacterial species-specific variability in their cytokine response to different *Lactobacilli*. For example, *L. rhamnosus, Lactobacillus plantarum* and *Lactobacillus casei* induce only weak cytokine production in DCs (11, 12, 28, 77, 203), while *Lactobacillus acidophilus, Lactobacillus johnsonii, Lactobacillus reuteri* and *Lactobacillus gasseri* efficiently induce the production of pro-inflammatory cytokines and IL-12 in DCs (20, 117, 203). Importantly, T cell polarization induced by *Lactobacilli*-stimulated DCs has been shown to correlate with the ability of different species to induce DC activation and cytokine production. *Lactobacillus* species that do not induce DC cytokine production result in non-Th1/Th2 or tolerogenic T cell response while IL-12 inducing species enhance Th1 polarization (11, 12, 117, 168). Although these *in vitro* studies do not directly give mechanistic explanation to the possible systemic immunomodulation by probiotic bacteria they clearly demonstrate that probiotic bacteria modulate APC functions and the development of adaptive immune responses. Moreover, the requirement of intestinal microbiota for the development and maintenance of properly functioning intestinal immune system is well recognized (97, 130).

Only few studies have addressed the recognition and signal transduction induced by lactobacilli. Purified LTAs from several *Lactobacillus* species induce macrophage activation via TLR2 (104). However, in a transfection model *L. reuteri*, *L. casei* or *L. plantarum* did not induce TLR-mediated NF-κB activation (168). Binding of *L. casei* and *L. plantarum* on mo-DCs has been shown to be mediated by DC-SIGN (168). Moreover, the DC-SIGN-mediated signals were required for the proliferation of regulatory T cells induced by *L. casei* and *L. plantarum*-stimulated mo-DCs (168).

3 AIMS OF THE STUDY

Macrophages and dendritic cells control innate antimicrobial responses and the subsequent development of adaptive immunity. Activated macrophages and dendritic cells produce cytokines and chemokines, which have an essential role in regulating both innate and adaptive immune responses. In this thesis work the activation and cytokine/chemokine response of different antigen presenting cell types in response to pathogenic and non-pathogenic Gram-positive bacteria was analyzed. Another key question of this study was the role of pro-inflammatory cytokines in the regulation of bacteria and influenza A virus-induced inflammatory chemokine gene expression.

The specific aims of this study were:

• To characterize the chemokine gene expression profile of human monocyte-derived macrophages stimulated with pathogenic *S. pyogenes* and non-pathogenic *L. rhamnosus*.

• To analyze the activation of monocyte-derived DCs and blood primary DCs in response to Gram-positive pathogenic and non-pathogenic bacteria

• To characterize the role of pro-inflammatory cytokines in the regulation of inflammatory chemokine gene expression in macrophages and epithelial cells

4 MATERIALS AND METHODS

4.1   Cell culture

Most of the experiments in this thesis were performed with primary human leukocytes. These cells were isolated form leukocyte-rich buffy coats obtained from Finnish Red Cross Blood Transfusion Service. In each experiment cells from two to four donors were used. Macrophages and mo-DCs were differentiated *in vitro* from monocytes, while blood primary mDCs and pDCs were isolated from PBMCs by using magnetic separation and stimulated subsequently *in vitro* with different microbes. The methods are described below in brief, detailed descriptions can be found in the original publications.

4.1.1   Isolation of PBMCs (I, II, III)

PBMCs were isolated by Ficoll density gradient centrifugation after which the mononuclear cell layer was collected and washed. Monocytes were separated by allowing them to adhere to plastic cell culture plates and the non-adherent cells were removed by washing with phosphate-buffered saline (PBS) solution. When mo-DCs were generated, monocytes were further purified by an additional Percoll gradient centrifugation step followed by magnetic depletion of T cells and B cells prior to cell adherence.

4.1.2   Differentiation of macrophages and DCs

Macrophages were cultured in serum-free macrophage medium (Gibco) supplemented with antibiotics, glutamine and 10 ng/ml of GM-CSF for 7d. Fresh medium was changed every two days. Mo-DCs were cultured in RPMI1640 medium (Sigma) supplemented with antibiotics, glutamine, 10 % fetal calf serum (FCS), 10 ng/ml of GM-CSF and 20 ng/ml of IL-4. Fresh medium was added every two days. After 6-7 d culture mo-DCs were mostly non-adherent and expressed DC-specific cell surface proteins.

4.1.3   Isolation of blood primary DCs and CD4$^+$ T lymphocytes

Blood primary DCs were isolated from PBMCs by using magnetic separation methods. pDCs were isolated by positive selection using BDCA4 dendritic cell isolation kit (Miltenyi Biotec). mDCs were isolated with BDCA1 DC isolation kit

(Miltenyi Biotec). Isolated pDC were CD123$^+$ BDCA2$^+$ double-positive and no CD11c expression was detected. mDCs were over 95% CD11c positive and showed no expression of pDC markers. pDCs were cultured in RPMI1640 medium supplemented with antibiotics, glutamine, 10 % FCS and 10 ng/ml of IL-3. mDCs were cultured in the same medium as mo-DCs. Naïve CD4$^+$ T cells were isolated from cord blood by negative selection using CD4$^+$ T cell isolation kit II (Miltenyi Biotec). The purity of CD4$^+$ cells was over 95 %.

4.1.4 Cell lines

The A549 lung adenocarcinoma cell line (ATCC CCL-185) was obtained from American Type Culture Collection. Cells were grown in plastic cell culture flasks in Eagle-MEM (Sigma) supplemented with antibiotics, glutamine and 10 % FCS.

4.2   Bacteria and viruses used (I, II, III, IV)

S. pyogenes serotype T1M1 (IH32030), isolated from a child with bacteremia was obtained from the collection of National Public Health Institute (Helsinki, Finland) and *L. rhamnosus* GG (ATCC 53103) was from Valio R&D (Helsinki, Finland). *S. enterica* serovar typhimurium (*S. typhimurium*) was obtained from Prof. Timo Korhonen. Bacteria were stored at -70°C and grown to logarithmic growth phase for stimulation experiments. Cells were stimulated with live bacteria unless otherwise indicated. Prior to stimulation, the bacteria were centrifuged and suspended to RPMI1640 medium and added to cells. Macrophages were stimulated at 1:1 bacteria:macrophage ratio, while 5:1 ratio was determined to be optimal for DCs.

Influenza A H1N1 (A/New Caledonia/20/99), influenza A H3N2 (A/Beijing/353/89), influenza B (B/Victoria/2/87) and Sendai (strain Cantell) viruses were grown in 11 day-old embryonated eggs. Cells were infected with MOIs indicated in each experiment and egg allantois fluid was used in the mock/control infections.

4.3   mRNA expression analyses (I, II, IV)

Cells from four blood donors were pooled and total cellular RNA was isolated by guanidium isothiocyanate/CsCl method or by using RNeasy RNA isolation kit (Qiagen). RNA was quantified photometrically, and samples containing equal amounts (10 µg) of RNA were size-fractionated on 1 % formaldehyde-agarose gels, and transferred to nylon membranes. The probes for Northern blot analysis were labeled with [α-^{32}P]dATP (3000 Ci/mmol) using random primed DNA labeling kit.

Hybridizations were performed in Ultrahyb buffer (Ambion). After hybridization the membranes were washed and exposed to Kodak X-Omat AR film at -70°C with intensifying screens. Details about the cDNA probes used can be found in the original publications. Equal sample loading was controlled by staining rRNAs with ethidium bromide.

4.4 Protein expression analyses

4.4.1 Cytokine and chemokine measurements (I, II, III, IV)

For the analysis of cytokine and chemokine production cell culture supernatants from different donors were pooled or analyzed separately (indicated in each original publication). Cytokine and chemokine levels were analyzed from cell culture supernatants by a sandwich-ELISA method. The antibody pairs and standards were obtained from BD Pharmingen, R&D Biosystems and Biosite (Details in the original publications). In publication III, the expression of pro-inflammatory and Th1/Th2 cytokines was measured by using FlowCytomix bead assay (Bender Medsystems) and flow cytometry.

4.4.2 Flow cytometry (II, III)

For flow cytometric analysis, the cells from three to four blood donors were pooled after stimulation experiments. Cells were washed with PBS and fixed with 3 % paraformaldehyde. The expression of co-stimulatory molecules and pDC/mDC specific cell surface markers was analyzed by using FITC- or PE-conjugated antibodies and respective isotype controls. Cells were analyzed with FACScan flow cytometer and Cellquest software (BD Biosciences).

4.4.3 Western blotting (IV)

Cells from different donors were pooled and were lysed into TN-buffer containing protease inhibitors. Equal amounts of protein were separated on SDS-PAGE and transferred onto membranes. Membranes were stained in 5 % nonfat milk. Details about the antibodies used can be found in the original publications. Proteins were visualized by using the enhanced chemiluminescence system (Amersham).

4.5   Transcription factor activation analysis (IV)

Transcription factor activation was studied by DNA affinity binding assay. Cells were lysed and samples were treated as described by Rosen et al (149). The oligonucleotides used were ISRE and NF-κB elements from CXCL10 promoter. The forward oligonucleotide was 5′-biotinylated and a *BamHI* site was added as a spacer between the biotin and transcription factor binding sequence. Detailed sequences are described in IV. Oligonucleotides were annealed in 0.5 M NaCl and incubated with streptavidin-agarose beads (Pierce) at +4°C for 2 h. The unbound oligonucleotide was washed, after which equal amounts of protein samples were incubated with the oligonucleotide bound agarose beads for 2 h at +4°C. After washings, the bound proteins were boiled in SDS sample buffer and separated on SDS-PAGE followed by Western blotting.

5   RESULTS AND DISCUSSION

The aim of this thesis work was to analyze the activation and regulation of inflammatory cytokine and chemokine production in different cell types. More specifically, the ability of pathogenic *S. pyogenes* and non-pathogenic *L. rhamnosus* to activate human monocyte-derived macrophages and mo-DCs was first evaluated (I, II). *S. pyogenes* was found to be an extremely potent activator of cytokine and chemokine production, while *L. rhamnosus* induced a more limited response especially in mo-DCs. In subsequent experiments the ability of *S. pyogenes* to activate blood primary DC subtypes was analyzed (III). *S. pyogenes* stimulation induced effective maturation and cytokine production of primary DCs. Interestingly, also pDCs that have been previously thought as "virus-restricted" DCs, were activated by *S. pyogenes*. Another key question of the present work was the role of pro-inflammatory cytokines in microbe-induced chemokine production. The results demonstrate that pro-inflammatory cytokines enhance microbe-induced production of certain inflammatory chemokines during infection and that pro-inflammatory cytokines can prime normally unresponsive cells for efficient microbe-induced chemokine production (I, IV).

5.1   Macrophage chemokine response to Gram-positive bacteria (I)

5.1.1   *S. pyogenes* and *L. rhamnosus*-induced chemokine production in macrophages

Tissue resident macrophages have an important role in innate immune response against microbial pathogens. Activated macrophages ingest and kill microbes by phagocytosis and produce a large variety of cytokines and chemokines. Tissue macrophages are in the first line of anti-microbial defence and thus macrophage-derived chemokines have a crucial role in recruiting other effector cells to the site of inflammation. Previous observations in our laboratory have shown that *S. pyogenes* and *L. rhamnosus* induce the production of pro-inflammatory cytokines in macrophages and PBMCs (111, 112, 114). However, when this thesis work was initiated the ability of Gram-positive bacteria to induce inflammatory chemokine production had not been systematically analyzed.

In our experimental setting human monocyte-derived macrophages were stimulated with live bacteria and the chemokine production was measured at mRNA and protein level. *S. pyogenes*- and *L. rhamnosus*-stimulated macrophages produced

similar patterns of inflammatory chemokines. These included CCL2, CCL3, CCL5, CCL7, CCL20 and CXCL8 (Figs. 1 and 2 of I). Subtle differences in the induction kinetics of these chemokines were observed at the mRNA level between *S. pyogenes* and *L. rhamnosus*. However, at 24 h the protein levels measured from cell culture supernatants were similar between the two bacteria. The inflammatory chemokines produced by *S. pyogenes*- and *L. rhamnosus*-stimulated macrophages attract several effector cell types. CCL2, CCL3, CCL5 and CCL7 recruit monocytes, granulocytes and T cells, while CXCL8 attracts neutrophils (98, 124, 135). The production of CCL20 by macrophages has not been previously reported but it may have a critical role in the activation of adaptive immune response since this chemokine attracts immature DCs (51, 169). Bacteria-induced inflammatory cytokine and chemokine response of monocyte/macrophages has recently been addressed in several global gene expression analyses. Macrophages and mononuclear cells stimulated with Gram-positive or Gram-negative bacteria were found to express several inflammatory chemokines including CCL2, CCL3, CCL4, CCL5, CXCL8 together with pro-inflammatory cytokines (34, 125). In another report purified TLR2 and TLR4 ligands were shown to induce the production of CC-chemokines in human monocytes and in a monocytic cell line (138). In conclusion, these results demonstrate that macrophages produce a large number of cytokines and chemokines in response to both pathogenic and non-pathogenic bacteria. Macrophage-derived cytokines and chemokines enhance local immune defence and efficiently recruit additional effector cells to the inflammation site.

5.1.2 Direct and indirect mechanisms of chemokine gene activation in bacteria-stimulated macrophages

Certain important differences in the macrophage chemokine response were, however, noted between *L. rhamnosus* and *S. pyogenes*. Only *S. pyogenes* induced efficient production of CXCL9 and CXCL10, which are chemoattractants for Th1 and NK cells (66, 121). The difference in the production of CXCL9 and CXCL10 had also a clear functional effect as the migration of Th1 cells was markedly higher in response to supernatants from *S. pyogenes*-stimulated macrophages compared to supernatants from *L. rhamnosus*-stimulated macrophages (Fig. 6 of I). Since *S. pyogenes* is able to invade and exist intracellularly in epithelial cells and neutrophils (118), the production of CXCL9 and CXCL10 and resulting NK cell recruitment could have a role in enhancing the recognition and destruction of intracellular *S. pyogenes*.

In the next set of experiments the mechanisms involved in the different expression of CXCL9 and CXCL10 in *S. pyogenes* and *L. rhamnosus*-stimulated macrophages

were evaluated. The mRNA and protein expression data showed that these chemokines were induced with a slower kinetics compared to other inflammatory chemokines. This suggested that the expression of CXCL9 and CXCL10 could be induced indirectly i.e. via the production of pro-inflammatory cytokines. To test whether bacteria-induced chemokine production was direct or cytokine-mediated macrophages were stimulated with bacteria in the presence of protein synthesis inhibitor cycloheximide. These experiments revealed that the bacteria-induced expression of CCL2, CCL7, CXCL9 and CXCL10 mRNAs was at least partially dependent on ongoing protein synthesis (Fig. 3 of I). At the protein level the clearest difference between *L. rhamnosus* and *S. pyogenes* was observed in the production of CXCL9 and CXCL10 suggesting that only *S. pyogenes* induced the mediator(s) enhancing the production of these chemokines. Our previous studies have shown that *S. pyogenes* but not *L. rhamnosus* induces IFN-α/β production in macrophages (111). Moreover, the expression of CXCL9 and CXCL10 has been shown to be inducible by IFNs and TNF-α (131, 132). In light of this information macrophages were stimulated with *L. rhamnosus* and *S. pyogenes* in the presence of neutralizing antibodies against type I IFNs. Neutralization of IFN-α/β during bacterial stimulation decreased *S. pyogenes*-induced CXCL9 and CXCL10 mRNA expression demonstrating a role of type I IFNs in enhancing *S. pyogenes*-induced expression of these chemokines. IFN-α/β most likely acts by inducing the formation of ISGF3 complex, which can bind to ISRE element on the CXCL10 promoter. The combined results from cycloheximide and IFN-neutralization experiments show that bacteria-induced expression of CXCL9 and CXCL10 is mediated by both direct microbe-induced transcription factor activation and via positive feedback loop provided by IFN-α/β and possibly by other pro-inflammatory cytokines. However, IFN-α and especially TNF-α alone induced only low CXCL9 and CXCL10 mRNA expression in macrophages (Fig. 5 of I). When these cytokines were combined a strong synergistic induction was detected. Since CXCL9 and CXCL10 proximal promoters contain ISRE and NF-κB binding sites (131, 132) these results suggest that the activation of both NF-κB and STAT transcription factors, provided by TNF-α and IFN-α/β or microbial stimulation is required for efficient CXCL9 and CXCL10 expression. IFN-α/β mediated feedback loop has been shown to operate in LPS-stimulated murine macrophages by enhancing the expression of iNOS, MCP5 and CXCL10 (38, 187).

The finding that *S. pyogenes* is able to induce type I IFNs and IFN-inducible chemokines in macrophages is also of interest. The classical receptor for Gram-positive bacteria is TLR2, which does not activate TRIF-mediated signal transduction pathway leading to type I IFN production. Thus, purified TLR2 ligands do not induce IFN production in macrophages (187). Moreover, when Nau and coworkers compared the gene expression pattern of macrophages stimulated with

Gram-positive and Gram-negative bacteria, the most significant differences were observed in the expression of IFN-inducible genes, which were only induced by Gram-negative bacteria (126). These results suggest that *S. pyogenes* can induce macrophage activation and expression of IFN-inducible genes in a TLR2-independent fashion. In recent microarray analysis it was shown that the activation of cytokine and chemokine genes by Group B Streptococcus is largely TLR2-independent in macrophages. Bacteria-induced expression of CC-chemokines, CXCL10 and TNF-α was somewhat slower in TLR2 knockout mice but at late time points the expression levels were nearly similar compared to wild type mice (29). It is, however, likely that TLR2 contributes to streptococci-induced macrophage activation since neutralizing anti-TLR2 antibodies reduce *S. pyogenes*-induced NF-κB activation (Miettinen et al unpublished results). Also, LTA from Group B streptococci has been shown to induce NF-κB activation and TNF-α production via TLR2/6 heterodimer (57). The TLR2-independent macrophage activation of *S. pyogenes* could be mediated i.e. via TLR9, which recognizes unmethylated CpG-rich DNA found in bacterial and viral genomes. However, in our experimental setting macrophages do not express this receptor (113). Another possibility could be intracellular NOD2 receptor, which has been shown to mediate *S. pneumoniae*-induced NF-κB activation (136). However, NOD2 has not been shown to induce IRF or STAT activation or to induce the production of type I IFNs.

In conclusion, these results show that both pathogenic and non-pathogenic Gram-positive bacteria induce the production of inflammatory cytokines and chemokines in macrophages. Although there seems to be a "general proinflammatory cytokine set" composed of TNF-α, IL-6 and CC-chemokines, and this gene pattern is induced by Gram-positive and Gram–negative bacteria as well as TLR2/4 ligands, there are still major qualitative differences between bacterial species and strains in the gene expression profile induced. The differences probably indicate that some bacteria are able to activate intracellular signal transduction via multiple PRRs, which results in a broad expression of inflammatory genes. The results obtained here also demonstrate an important role of secreted pro-inflammatory cytokines in enhancing bacteria-induced chemokine expression by providing a positive feedback loop.

5.2 Activation of monocyte-derived DCs by Gram-positive bacteria (II)

5.2.1 *S. pyogenes* and *L. rhamnosus*-induced mo-DC maturation

When the DC study was initiated large body of evidence on the ability of TLR ligands and pathogenic microbes to induce DC activation was already available. However, far less was known on the ability of non-pathogenic bacteria, especially Gram-positive bacteria, to modulate DC functions. Thus, the methodology for DC isolation and culturing was set up. Previous studies had shown that the maturation of immature DCs results in significant changes in DC transcriptome, which renders DCs to efficient T cell activators. Microbe-stimulated DCs produce cytokines, chemokines, tissue remodeling enzymes and show enhanced expression of T cell-stimulating cell surface molecules (47, 64, 84). In this study the maturation of mo-DCs was characterized by analyzing the expression of cell surface co-stimulatory molecules and the production of cytokines and chemokines. To address the functional maturation of DCs, the effect of bacterial stimulation on DC endocytosis, which is known to be reduced during maturation, was analyzed.

Both *L. rhamnosus* and *S. pyogenes* enhanced the expression of CD80, CD83 and CD86 co-stimulatory molecules in a bacterial dose-dependent manner (Fig. 1 of II). *S. pyogenes*-stimulated DCs showed slightly higher expression of CD83 and CD86 compared to *L. rhamnosus*-stimulated cells. Enhanced expression of co-stimulatory molecules was seen after 12 h stimulation and it reached maximum levels at 24 h (unpublished data). *S. pyogenes*-stimulation also efficiently reduced the endocytosis of FITC-dextran by DCs (Fig. 5 of II). The reduction of DC endocytosis was less evident in *L. rhamnosus*-stimulated DCs suggesting that *L. rhamnosus*-stimulated DCs matured less well than *S. pyogenes*-stimulated DCs. In conclusion, *S. pyogenes*-stimulated DCs showed a mature phenotype while *L. rhamnosus*-stimulated DCs had a more "semi-mature" DC phenotype. These DCs have been suggested to promote tolerance (152, 172).

5.2.2 Distinct cytokine responses in *S. pyogenes* and *L. rhamnosus*-stimulated mo-DCs

When bacteria-induced cytokine and chemokine production was analyzed, a striking difference between *S. pyogenes* and *L. rhamnosus* was observed. Only *S. pyogenes*-stimulation resulted in the production of pro-inflammatory cytokines and chemokines from mo-DCs (Fig. 2 of II). This was in stark contrast with our previous

findings on PBMCs or macrophages where the production of most pro-inflammatory cytokines and chemokines was induced in a nearly similar fashion by both *S. pyogenes* and *L. rhamnosus* (111, 112, 114, and I).

S. pyogenes induced the production of Th1 recruiting CXCL9 and CXCL10 chemokines and the production of IL-12. Moreover, the expression of IL-23 and IL-27 subunits p19/p40 and p28/EBI3, respectively, was induced at mRNA level (Fig. 4 of II). These results suggested that *S. pyogenes*-stimulated DCs could initiate efficient Th1 polarization. This was later demonstrated to be the case in *S. pyogenes*-stimulated blood primary myeloid DCs which enhanced IFN-γ production from naïve CD4$^+$ T cells (Fig. 6 of III). Interestingly, we observed that mo-DCs stimulated with *S. pyogenes* produced IL-2. The ability of DCs to produce IL-2 has previously been shown only in murine DCs (45, 46). In our experimental setting the inducible IL-2 production was restricted to *S. pyogenes* and not detected in mo-DCs stimulated with *L. rhamnosus*, *S. typhimurium* or LPS (Figs. 2 and 4 of II and unpublished observations). DC-derived IL-2 has been shown to play a role in enhancing T cell proliferation and NK cell activation in murine models (46, 48). The ability of *S. pyogenes* to induce the production of IL-2 in addition to IL-12 is likely to play a role in the efficient Th1 polarization induced by *S. pyogenes*.

The finding that *L. rhamnosus* was unable to induce cytokine production in DCs was somewhat surprising as this bacterium was one of the best cytokine inducers in PBMCs (112, 114). Our results on the lack of cytokine response in *L. rhamnosus*-stimulated mo-DCs have been confirmed in recent reports (11, 12, 203). The inability of *L. rhamnosus* to induce cytokine production can not, however, be generalized to whole lactobacillus genus. For example *L. casei*, *L. reuteri* and *L. johnsonii* have been shown to efficiently induce TNF-α, IL-10, IL-12 and IL-18 in human mo-DCs (117, 203). In our ongoing experiments performed with more than ten different *Lactobacillus* and *Bifidobacteria* species we have observed that several Gram-positive bacteria from these genera are in fact efficient cytokine inducers in mo-DCs. *L. rhamnosus*-stimulated mo-DC showed a "semi-mature" phenotype. These DCs are partially matured but do not produce pro-inflammatory cytokines or IL-12. Moreover, these DCs have been suggested to be tolerogenic and promote the proliferation of regulatory T cells (152, 172). Thus, it could be speculated that *L. rhamnosus*-stimulated DCs may enhance tolerance instead of inflammatory Th1 response. Previous reports have shown that, depending on the *Lactobacillus* strain and experimental setting used, probiotic bacteria-stimulated DCs can induce the proliferation of regulatory T cells (55, 198) or promote Th1 polarization (117, 141). Most importantly, the polarization of T cell response correlates with the ability of bacteria to activate DCs. Since DCs are powerful modulators of the adaptive immunity and DCs are present in the gastrointestinal mucosal tissue, modulation of

DC functions could be one mechanism of the immunomodulatory effects of probiotic bacteria. These effects could include probiotic-induced activation and Th1 polarization of the immune system during early childhood or perhaps enhancement of intestinal tolerance. However, these intriguing mechanisms remain speculative as no direct experimental data is currently available.

5.2.3 *L. rhamnosus* and *S. pyogenes* differentially activate mo-DC transcription factor networks (unpublished results)

In order to elucidate the mechanisms behind the distinct ability of *S. pyogenes* and *L. rhamnosus* to induce mo-DC cytokine production we analyzed the transcription factor pathways activated by these bacteria. In macrophages both *L. rhamnosus* and *S. pyogene*s activate the NF-κB pathway (111). However, *S. pyogenes* is a more potent activator of STATs, IRF1 and the ISGF3 complex (111).

Similarly to macrophages, both *L. rhamnosus* and *S. pyogenes* activated the NF-κB pathway in mo-DCs (Fig. 2). DNA binding of the components of classical (p50/p65) and alternative (p52/RelB) NF-κB pathways was detected on CXCL10 NF-κB element (Fig. 2). These results also confirmed that the difference between bacteria in the ability to induce mo-DC activation is not due to the lack of interaction between *L. rhamnosus* and mo-DCs. A hallmark difference between bacteria was seen in the activation of IRFs and STATs. *S. pyogenes* induced the DNA binding of IRF1, IRF4, IRF7, IRF8, IRF9, STAT1 and STAT2 on CXCL10 ISRE element. In contrast, *L. rhamnosus* stimulation most prominently enhanced DNA binding of IRF4 while the activation of other IRFs and STATs was weak and only detected after 24 h stimulation. This is of interest, since IRF4 has recently been suggested to act as a negative regulator of TLR-mediated cytokine expression (62, 127). Thus, it could be speculated that *L. rhamnosus*-induced activation of IRF4 may reduce DNA binding of activating IRFs or STATs, which could limit cytokine and chemokine gene expression. Further studies are on the way to elucidate the contribution of differential IRF activation on distinct cytokine and chemokine response observed in *S. pyogenes* and *L. rhamnosus*-stimulated mo-DCs.
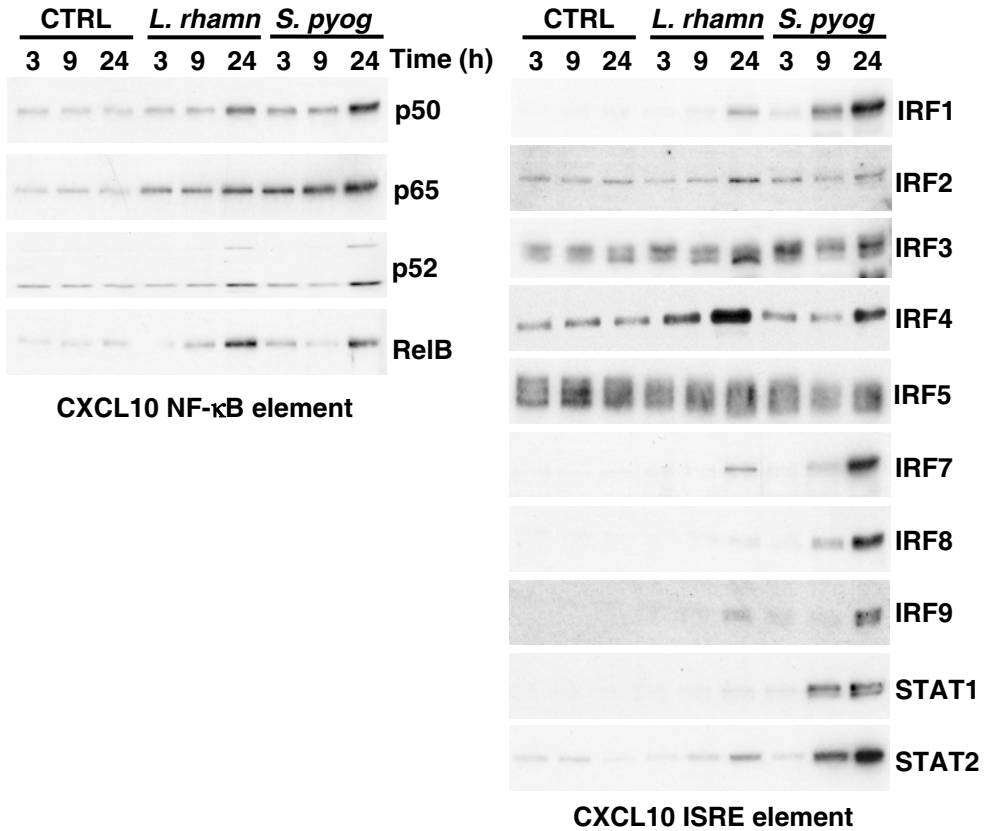
Figure 2. *S. pyogenes and L. rhamnosus-induced NF-κB, IRF and STAT activation in mo-DCs. DCs were stimulated with bacteria for the times indicated and the DNA binding of transcription factors was analyzed by oligonucleotide affinity binding assay by using NF-κB and ISRE elements from CXCL10 proximal promoter. The classical (p50/p65) and alternative (p52/RelB) NF-κB pathway was similarly activated by both bacteria. In contrast, IRFs and STATs were activated efficiently only by S. pyogenes.*

5.3 Activation of blood primary DCs by bacteria and influenza A virus (III)

Recent advances in cell isolation techniques and the discovery of novel DC subtype-specific molecules has enabled the isolation of primary human mDCs and pDCs from peripheral blood. Since our findings on macrophages and mo-DCs had shown that *S. pyogenes* is an extremely potent APC activator we broadened the analysis to primary mDCs and pDCs. Moreover, the possible involvement of pDCs in antibacterial defence has not been previously elucidated in detail. Early reports, however, suggested that Gram-positive *S. aureus* could activate pDCs (165).

In the first set of experiments the ability of bacteria to induce pDC activation was analyzed. LPS was used as a negative control since pDCs do not express TLR4 and are therefore unresponsive to LPS (93). In these preliminary experiments, we observed that *S. pyogenes* induced significant TNF-α production in pDCs whereas *L. rhamnosus*, Gram-negative *S. typhimurium* or LPS failed to induce TNF-α (Fig. 1 of III and data not shown). A well characterized pDC activator, influenza A virus, was a more potent TNF-α inducer than *S. pyogenes*. When DC maturation was analyzed, we observed that *S. pyogenes* and influenza A virus enhanced the expression of CD40, CD86 and MHCII in pDCs (Fig. 3 of III). At 24 h, influenza A virus induced the expression of co-stimulatory molecules better that *S. pyogenes* but at 48 h the expression levels were equal. Unstimulated mDCs already expressed high levels of co-stimulatory molecules and the expression of CD40, CD86 and HLAII was enhanced only marginally in response to *S. pyogenes*. mDCs were very sensitive to the cytopathic effects of influenza A virus, which was observed in the viability assay as well as in the inability of influenza A virus to enhance the expression of co-stimulatory molecules in mDCs (Figs. 2 and 3 of III).

Both *S. pyogenes*-stimulated pDCs and mDCs produced pro-inflammatory cytokines TNF-α and IL-6 together with CCL3 and CXCL8 (Figs. 4 and 5 of III). However, certain differences in the cytokine profiles of mDCs and pDCs were also observed. Most importantly, only mDCs produced IL-10 and IL-12 in response to *S. pyogenes*. Although early reports suggested that pDCs are able to produce IL-12, more recent studies have shown that human pDCs do not produce IL-12 (69, 70, 120). The absence of IL-12 in *S. pyogenes*-stimulated pDC supernatants also confirms that *S. pyogenes*-induced production of other inflammatory cytokines and chemokines is not mediated by contamination with myeloid cells. Another difference was observed in the production of IFN-α, which was only detected in influenza A virus-stimulated pDCs and mDCs. However, influenza A virus-induced cytokine response was generally very low in mDCs, which is associated with the high susceptibility of these cells to influenza A virus infection.

Our observations in *S. pyogenes*-stimulated macrophages and mo-DCs demonstrated that this bacterium induces a strong Th1 type cytokine response including the production of IL-12 and IL-27 and Th1 recruiting chemokines CXCL9 and CXCL10 (111, I and II). In line with this data we detected Th1 type IFN-γ production from naïve T cells co-cultured with *S. pyogenes*-stimulated mDCs and pDCs (Fig. 6 of III). *S. pyogenes*-stimulated pDCs also induced the production of IL-10 from T cells. It could be speculated that pDCs can polarize T cells towards mixed Th1 and regulatory T cell population. It is likely that other mediators apart from IL-12 and IFN-α regulate pDC-induced Th1 polarization, since these cytokines were not produced by *S. pyogenes*-stimulated pDCs. Virus- or CpG-DNA-stimulated pDCs have been previously shown to induce the proliferation of Th1 cells and regulatory T cells with $CD4^+CD25^+$ phenotype (16, 78, 120).

The receptor mediating *S. pyogenes*–induced pDC responses remains to be further characterized. pDCs do not express TLR2, the prototype receptor for Gram-positive bacteria (72, 74). An obvious receptor candidate is TLR9 which recognizes CpG-rich DNA found in bacteria and DNA viruses (7, 93). However, *S. pyogenes*-induced TNF-α production was unaffected when inhibitory CpG oligonucleotides or non-specific TLR9 inhibitor chloroquine was used during stimulation (unpublished observations). Other alternatives for the *S. pyogenes* receptor include scavenger or lectin-type receptors, which are broadly expressed in pDCs (9, 93). Although the role of these receptors in regulating pDC maturation and cytokine production has so far remained uncharacterized, recent reports have show that these receptors, including Dectin-1 and DC-SIGN, activate intracellular signalling and cytokine production in other cell types (14, 148, 189).

Certain differences in *S. pyogenes*-stimulated mDCs and mo-DCs were observed. In our experimental setting *S. pyogenes*-stimulated mDCs produced high levels of IL-10 within 24-48 h, while the IL-10 production in mo-DCs was low and detectable only after 48 h *S. pyogenes* stimulation (unpublished observations). Unexpectedly, no IL-2 or CXCL10 was detectable in mDC supernatants although these mediators were produced by *S. pyogenes*-stimulated mo-DCs. Thus, although similar in many aspects, mDCs and mo-DCs have also differences in their microbial responses.

In conclusion, these results show that human primary DCs are efficiently activated by *S. pyogenes*. Interestingly, also pDCs, which have previously been thought to be "virus-specific" DCs, matured and produced inflammatory cytokines and chemokines in response to *S. pyogenes*. Thus, the different human DC subtypes may have more functional flexibility in their bacterial responses than previously anticipated. These results also demonstrate that *S. pyogenes* efficiently activates several APC types, including macrophages, mo-DCs, mDCs and pDCs, and promotes the production of inflammatory cytokines and chemokines. The activation

several APCs may provide effective means for the host to control *S. pyogenes* infections. However, the high potency of *S. pyogenes* to induce cytokine and chemokine production may also play a role in severe group A streptococcal infections, which are characterized by extensive inflammation and tissue damage. *S. pyogenes*-induced excessive APC activation could also contribute to the development of post-streptococcal autoimmune diseases such as rheumatic heart disease and psoriasis (5, 8).

5.4 Regulation of influenza A virus-induced chemokine gene expression by pro-inflammatory cytokines (IV)

The results from publication I clearly demonstrated a role of pro-inflammatory cytokines, especially that of type I IFNs, in enhancing bacteria-induced chemokine production (I). In that setting type I IFNs and/or other pro-inflammatory cytokines were likely to directly activate transcription factors and enhance chemokine production during bacterial stimulation. Pro-inflammatory cytokines, however, also increase the expression of several transcription factors and PRRs, which could promote microbial recognition and signal transduction. To evaluate this aspect of pro-inflammatory cytokines in the regulation of chemokine production, we studied the effect of TNF-α and IFN-α priming on influenza A virus-induced chemokine production in human lung epithelial cells.

5.4.1 Influenza A virus-induced chemokine production and transcription factor activation

When A549 lung epithelial cells were infected with influenza A virus the expression of inflammatory chemokine genes was relatively weak and detected only when the highest viral doses were used (Figs. 1 and 2 of IV). To test the effect of cytokine priming, cells were treated with TNF-α or IFN-α prior to influenza A virus infection. After cytokine priming influenza A virus-induced expression of CCL2, CCL5, CXCL8 and CXCL10 was significantly enhanced (Fig. 3 of IV). Most importantly, cytokine priming enhanced the DNA binding of IRF1, IRF3 and IRF7 on CXCL10 ISRE element and NF-κB binding on CXCL10 NF-κB element. Without cytokine pre-treatment influenza A virus induced DNA binding of IRFs was undetectable and NF-κB DNA binding was only seen at 24 h post infection (Fig. 5 of IV).

5.4.2 Mechanisms of the enhanced chemokine response by cytokine pre-treatment

The above results clearly showed that TNF-α and IFN-α pre-treatment enhances virus-induced chemokine production. We hypothesized that this could be mediated by enhanced expression of signal transduction components and/or PRRs. Previous studies have shown that the expression of IRF1, IRF7 and STATs is IFN-inducible (4, 87, 100). Moreover, the expression of many TLRs and the recently described intracellular dsRNA sensor RIG-I is regulated by IFNs (113, 202). Similarly to these results, TNF-α and/or IFN-α enhanced the expression of IRF1 and IRF7 and their upstream components IKKϵ and RIG-I in A549 cells (Fig. 4 of IV). Another mechanistic explanation in virus model could be that TNF-α or IFN-α reduce virus replication and limit the expression of viral proteins that may down-regulate cytokine and chemokine production. For example, influenza A virus encodes non-structural (NS)1 protein which interferes with host cell mRNA processing and inhibits RIG-I-dependent activation of intracellular signalling (128, 140). However, no significant reduction in viral gene expression was observed at mRNA or at protein level in cytokine pre-treated cells compared to untreated cells (Figs. 3 and 5 of III). The cytokine pre-treatment also enhances TLR ligand-induced IFN production (167), which further suggests that the mechanism of cytokine pre-treatment relies on enhanced expression of PRRs and signal transduction components, not on mere inhibition of virus replication.

In conclusion these results show that pro-inflammatory cytokines can render normally unresponsive cells to high chemokine producers upon viral infection. The cytokine priming and the resulting increased chemokine response most likely enhances the migration of effector leukocytes to the site of inflammation and promotes more efficient resolution of the inflammation. Although influenza A virus induces only low IFN production in epithelial or endothelial cells, APCs and especially pDCs produce high levels of IFN-α and TNF-α in response to this virus (16, 21, Fig. 4 of III). Since APCs reside in close proximity to the epithelium, APC-derived pro-inflammatory cytokines may provide cytokine priming to epithelial cells also *in vivo*. It should, however, be noted that the effect of cytokine pre-treatment also depends on the stimulus in question. Although TNF-α and/or IFN-α enhance the inflammatory cytokine and chemokine production in TLR ligand-stimulated or virus-infected epithelial cells, macrophages and dendritic cells (137, 166, 167, and IV) the cytokine priming does not enhance the low cytokine and chemokine response detected in *L. rhamnosus*–stimulated mo-DCs (unpublished data).

6 CONCLUDING REMARKS

Macrophages and dendritic cells have an important role in the anti-microbial defence. These cells are armed for efficient microbial detection and, upon activation, macrophages and DCs produce cytokines and chemokines, which activate innate and adaptive immune responses. In this thesis work the ability of pathogenic *S. pyogenes* and non-pathogenic *L. rhamnosus* to activate inflammatory cytokine and chemokine gene expression in macrophages and DCs was compared. Differences between cell types as well as between pathogenic and non-pathogenic bacteria were observed.

Bacteria-stimulated macrophages produced similar core set of chemokines in response to *S. pyogenes* and *L. rhamnosus*. These included CXCL8 and several CC-chemokines, which enhance the recruitment of monocytes, neutrophils, immature DCs and T cells. Thus, macrophages activated by pathogenic or non-pathogenic Gram-positive bacteria efficiently enhance the local antimicrobial response by promoting the chemotaxis of other effector cells to the site of inflammation. Certain differences in the macrophage chemokine response between *S. pyogenes* and *L. rhamnosus* were, however, observed. Only *S. pyogenes*-stimulated macrophages produced inflammatory chemokines CXCL9 and CXCL10, which specifically attract Th1 and NK cells. The production of these chemokines and the recruitment of NK cells may have a role in enhancing the recognition and destruction of intracellular *S. pyogenes*.

While macrophages were activated by both *S. pyogenes* and *L. rhamnosus*, DCs showed a clearly distinct response against pathogenic and non-pathogenic bacteria. *S. pyogenes* induced efficient mo-DC maturation accompanied with a strong cytokine and chemokine response. *S. pyogenes*-stimulated mo-DCs produced pro-inflammatory cytokines, IL-12 family cytokines and Th1 recruiting chemokines CXCL9 and CXCL10. *S. pyogenes* also activated blood primary DCs and the *S. pyogenes*-stimulated blood primary DCs promoted Th1 polarization of naïve CD4^{+} T cells. Interestingly, *S. pyogenes* was also found to activate pDCs, which have been regarded as "virus-specific" DCs. Thus, *S. pyogenes* is a very potent activator of several human DC types and the human DC system may have more flexibility in the antibacterial responses than previously thought. The high potency of *S. pyogenes* to activate several immune cell types and to induce cytokine/chemokine production may be both beneficial and harmful for the host. Under normal conditions, rapid detection of *S. pyogenes* and the resulting activation of the immune response is likely to efficiently limit the spread of this infection. However, in some cases *S. pyogenes* infection may result in uncontrolled and excessive activation of the

immune response, which may lead to a severe tissue damage or to the development of autoimmune diseases.

In contrast to *S. pyogenes*, mo-DCs stimulated with non-pathogenic *L. rhamnosus* showed a "semi-mature" phenotype characterized by up-regulation of co-stimulatory markers but low or undetectable cytokine and chemokine production. The induction of tolerogenic DCs, which lack inflammatory cytokine response, may provide one explanation for the symbiotic coexistence between host and the commensal microflora. Moreover, partial but non-inflammatory activation of innate immune response may contribute to the suggested immunomodulatory effects of probiotic bacteria.

In conclusion, although macrophages and DCs share several common antimicrobial functions the responses against pathogenic and non-pathogenic bacteria were found to differ markedly. The results obtained in the present study suggest that tissue resident macrophages are efficiently activated by any bacteria, be that pathogenic or non-pathogenic. In contrast, DCs may be able to discriminate between pathogenic and non-pathogenic bacteria and therefore mount the adaptive immune response towards inflammation or tolerance/homeostasis (summarized in Fig. 3).

Another key question of this thesis work was to analyze the role of pro-inflammatory cytokines in the regulation of microbe-induced chemokine expression. Two key mechanisms were observed. 1) During microbial infection the produced pro-inflammatory cytokines were found to directly enhance the expression of certain chemokines by increasing transcription factor activation. This was observed in *S. pyogenes*-stimulated macrophages, where bacteria-induced production of type I IFNs enhanced the production of CXCL9 and CXCL10 chemokines. 2) Another important role of pro-inflammatory cytokines in enhancing microbe-induced chemokine production was seen in the priming experiments. Pre-treatment of epithelial cells with TNF-α or IFN-α significantly enhanced influenza A virus-induced transcription factor activation and chemokine response. Pro-inflammatory cytokines were found to enhance the expression of PRRs and transcription factors, thereby rendering epithelial cells for enhanced microbial detection and signal transduction. These mechanisms are summarized in Figure 4.
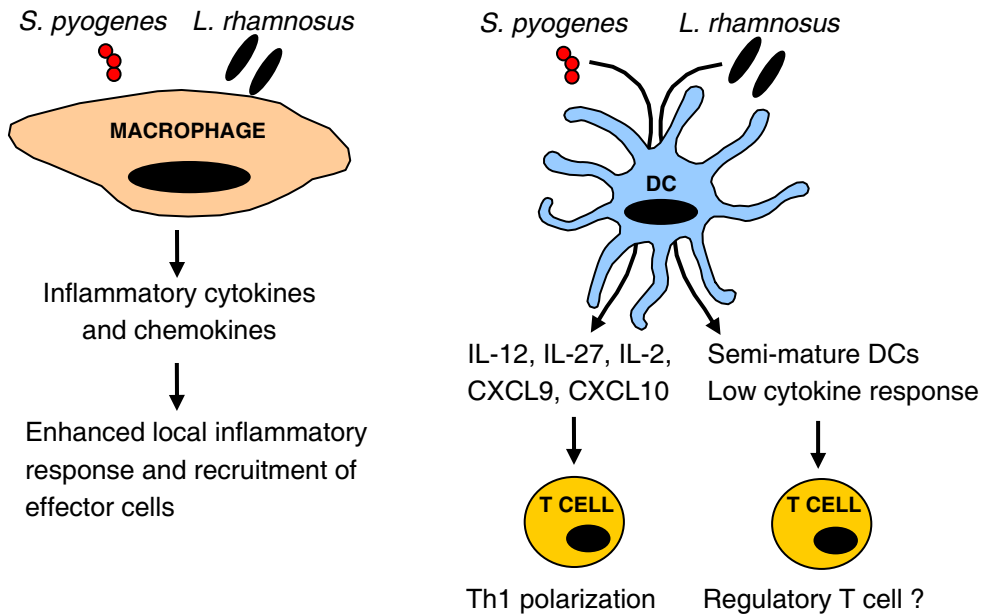
Figure 3. *Distinct activation of macrophages and DCs in response to pathogenic and non-pathogenic Gram-positive bacteria. Both pathogenic Streptococcus pyogenes and non-pathogenic Lactobacillus rhamnosus induce the production of inflammatory cytokines and chemokines in macrophages. This results in the enhancement of local immune response and recruitment of effector cells to the site of inflammation. In contrast, DCs may be able to discriminate between pathogenic and non-pathogenic bacteria, which results in the development of fully mature cytokine-producing DCs (S. pyogenes) or semi-mature tolerogenic-like DCs (L. rhamnosus). The differential DC maturation may lead to distinct T cell responses supporting inflammation or tolerance.*
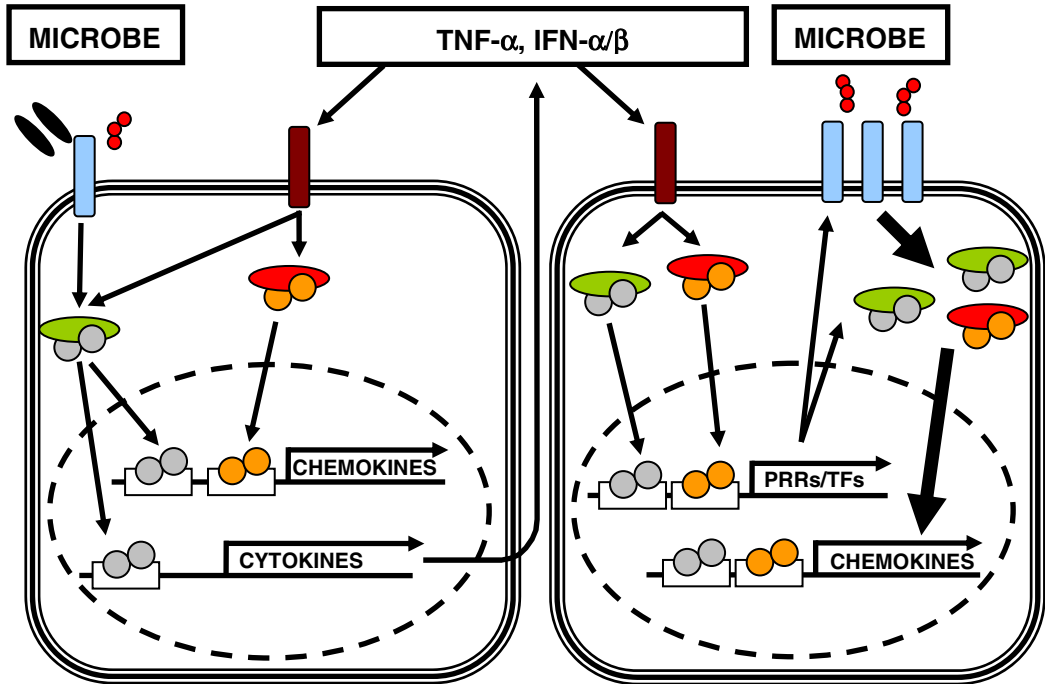
Figure 4. *Role of pro-inflammatory cytokines in the regulation of microbe-induced chemokine production. Microbe detection results in transcription factor activation, which leads to the production of pro-inflammatory cytokines such as TNF-α and type I IFNs (left cell). Secreted cytokines bind to their cell surface receptors, which further activates intracellular signalling. This results in enhanced expression of chemokines and other immunoregulatory genes during infection. Alternatively, TNF-α and IFN-α may induce the expression of PRRs and transcription factors (TFs) in uninfected cells (right cell). This renders cells for efficient microbe recognition, intracellular signalling and chemokine production upon infection.*

7 ACKNOWLEDGEMENTS

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