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Sinikka Latvala

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RESEARCH

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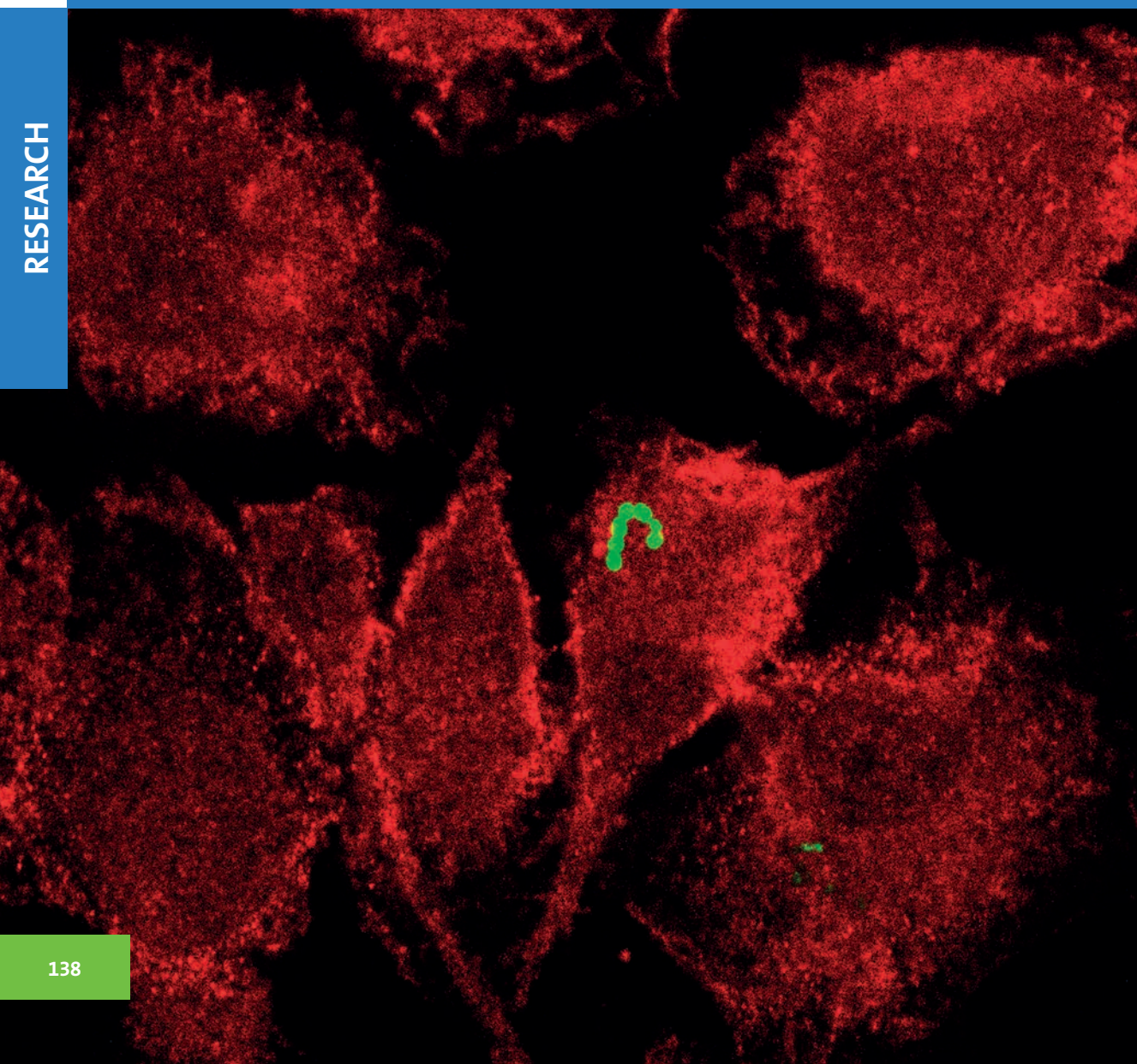

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Activation of Innate Immune Responses by Non-pathogenic and Pathogenic Bacteria in Human Leukocytes

ACADEMIC DISSERTATION

To be presented for public examination with the permission of the Faculty of Biological and Environmental Sciences, University of Helsinki, in the Auditorium 1041 of Biocenter 2, Viikinkaari 5, Helsinki on November 21st 2014 at 12 noon.

Virology Unit, Department of Infectious Disease Surveillance and Control, National Institute for Health and Welfare, Helsinki, Finland
and
Division of General Microbiology, Department of Biosciences, University of Helsinki, Finland

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Cover photo: Macrophages (red) reaching for *S. pyogenes* bacteria (green), immunofluorescence image by S&S.

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Abstract

Sinikka Latvala, Activation of Innate Immune Responses by Non-pathogenic and Pathogenic Bacteria in Human Leukocytes. National Institute for Health and Welfare (THL), Research 138. 86 pages. Helsinki, Finland 2014. ISBN 978-952-302-312-3 (printed); ISBN 978-952-302-313-0 (online publication)

The human body is continuously interacting with microbes. Cells of the immune system protect the host from microbes some of which can occasionally cause diseases. Macrophages and dendritic cells (DC) play a central role in innate and adaptive immune responses and they are activated in response to microbial stimulation. Macrophages function mainly locally in peripheral tissues taking up and destroying microbial pathogens. They are also important in maintaining inflammatory responses in inflamed tissues. DCs take up microbes which triggers DC maturation and migration of the cells into local lymph nodes, where antigen presentation to naïve T cells and induction of adaptive immune responses takes place. During activation these cells produce cytokines, soluble mediators that regulate host immune responses. Recognition of microbes is regulated by multiple host cell surface and intracellular receptor systems which after recognition of microbes or their structural and genetic components activate intracellular signalling pathways ultimately leading to the expression of genes involved in immune responses.

In this thesis the interactions between human leukocytes and various bacteria were studied using human primary macrophages and DCs. The innate immune responses induced by probiotic and other non-pathogenic Gram-positive bacteria, some of which are part of the normal gastrointestinal microbiota, were compared with the responses induced by a pathogenic bacterium *Streptococcus pyogenes* (group A streptococcus, GAS). The differences in the gene expression profiles of cytokine and chemokine genes and the activation of different signalling pathways were analysed. Also the anti-inflammatory potential of different bacteria was evaluated to predict their immunomodulatory capacity for potential clinical use.

Macrophages and DCs were compared in their cytokine responses to different non-pathogenic bacteria. Although both cell types effectively destroy invading microbes the cytokine patterns produced by the two cell types in response to bacterial stimulation varied significantly. Macrophages responded to bacterial stimulation in a nearly similar fashion whether the bacteria had probiotic, non-pathogenic, or pathogenic characteristics. Interestingly, DCs showed a much wider variability in their responses to different bacteria. Some non-pathogenic bacteria did not induce detectable levels of cytokines in DCs while others were even more efficient than *S. pyogenes* in inducing cytokine responses. DC maturation marker molecules were

induced in response to interaction with non-pathogenic bacteria with relatively weak cytokine production patterns. This led to a semi-mature DC phenotype, which could be involved in tolerogenic responses seen in host cell interactions with commensal bacteria.

The other focus of this work was to evaluate the role of dynamin-dependent endocytosis and streptococcal virulence factors, streptolysins, in the immune responses induced by *S. pyogenes* in macrophages. Streptolysins were not critical for the inflammatory responses induced by this bacterium, suggesting a cooperation of various virulence factors in bacterial pathogenesis.

Taken together, this work provides better understanding of the functions of the innate immune system upon contact with non-pathogenic and pathogenic bacteria. The results may provide useful information for the interpretation of the results obtained from probiotic clinical trials and help to select new candidate probiotic strains for clinical use. In addition, this work identifies new details on the mechanisms of *S. pyogenes* -induced inflammatory responses.

Keywords: cytokine, dendritic cell, macrophage, innate immunity, bacterium

Tiivistelmä

Sinikka Latvala, Innate Immune Responses Induced by Non-pathogenic and Pathogenic Bacteria in Human Leukocytes [Ei-patogeenisten ja patogeenisten bakteerien aiheuttama luontaisen immuniteetin aktivoituminen ihmisen leukosyyteissä]. Terveyden ja hyvinvoinnin laitos. Tutkimus 138. 86 sivua. Helsinki, Finland 2014. ISBN 978-952-302-312-3 (painettu); ISBN 978-952-302-313-0 (verkkojulkaisu)

Elimistön solut, erityisesti epiteelisolut ja immuunijärjestelmän solut, sekä epiteelipinnoilla elävät bakteerit ovat jatkuvasti vuorovaikutuksessa toistensa kanssa. Osa mikrobeista on harmittomia, jopa hyödyllisiä, toiset puolestaan voivat aiheuttaa tauteja päästessään elimistöön. Ihmisen immuunipuolustus voidaan jakaa luontaiseen ja hankittuun immuniteettiin. Luontainen immuniteetti pyrkii tuhoamaan elimistölle vaaralliset mikrobit nopeasti syöjäsolujen kuten makrofagien ja antigeeneja esittelevien dendriittisolujen toimesta. Nämä solut tunnistavat bakteereita erilaisten hahmontunnistusreseptoreiden välityksellä ja tuottavat runsaasti sytokiineja, jotka toimivat välittäjämolekyyleinä ohjaten sekä luontaista että hankittua immuunivastetta. Tuotetut sytokiinit riippuvat kohdatun mikrobin tai antigeenin ominaisuuksista sekä niihin reagoivasta solutyypistä. Sytokiinituotanto on tarkkaan säädeltyä ja mikrobin tai antigeenin virheellinen tunnistus voi johtaa joko immuunivasteen tehottomaan tai liian voimakkaaseen aktivoitumiseen.

Tässä työssä on tutkittu ihmisen leukosyyttien ja grampositiivisten bakteerien välisiä vuorovaikutussuhteita käyttäen mallina terveen ihmisen veren monosyyteistä erilaistettuja dendriittisoluja ja makrofageja. Työssä on havaittu selviä eroja probioottien, potentiaalisten probioottien ja patogeenisen *Streptococcus pyogenes* -bakteerin kyvyssä aktivoida immuunivasteita. Myös bakteerien anti-inflammatorinen potentiaali määritettiin niiden mahdollisten kliinisten vaikutusten ennustamiseksi.

Bakteereilla stimuloitujen dendriittisolujen kypsymistä, aktivaatiota ja sytokiinituotantoa verrattiin makrofageissa syntyviin vasteisiin. Pyrkimyksenä oli havaita mahdollisia eroja bakteerien kyvyssä aktivoida ihmisen immuunijärjestelmän soluja. Ei-patogeeniset bakteerit saivat aikaan dendriittisolujen kypsymisen ja kostimulatoristen molekyylien ilmentymisen solujen pinnalla, mutta niiden kyky aktivoida sytokiinituotanto vaihteli huomattavasti. Osa bakteereista ei aktivoinut sytokiinituotantoa lainkaan, toiset puolestaan aktivoivat sen voimakkaammin kuin patogeeninen *S. pyogenes* -bakteeri. Jotkut tutkituista bakteereista saivat aikaan dendriittisolujen osittaisen kypsymisen. Tällaiset solut ilmentävät kostimulatorisia molekyylejä, mutta eivät tuota sytokiineja, ja niiden uskotaan osallistuvan tolerogeenisten vasteiden syntymiseen. Tämä saattaa selittää

elimistön ja normaalin mikrobiston välistä toleranssia. Makrofageissa puolestaan kaikki tutkitut bakteerit saivat aikaan voimakkaan tai suhteellisen voimakkaan inflammatorisen vasteen. Tämä viittaa siihen, että bakteerin taudinaiheuttamiskyvyllä ei ehkä ole niin suurta merkitystä makrofageissa syntyvän luontaisen immuunivasteen kannalta.

Tutkimuksessa selvitettiin myös dynamiinivälitteisen endosytoosin osuutta puolustusvasteen synnyssä sekä verrattiin villityyppisen *S. pyogenes* –bakteerin ja streptolysiinivirulenssitekijöiden osalta puutteellisten bakteerien kykyä aktivoida makrofageja. Tutkimuksessa havaittiin, että streptolysiinit eivät ole välttämättömiä *S. pyogenes* -bakteerin aikaansaamien inflammatoristen vasteiden synnyssä.

Väitöskirjatyöstä saatu tieto auttaa ymmärtämään luontaisen immuunijärjestelmän toimintaa elimistön kohdatessa erilaisia bakteereita. Saatua tietoa voidaan käyttää hyödyksi tutkittaessa probioottisten bakteerien aikaansaamia terveysvaikutuksia ja niiden taustalla olevia mekanismeja sekä valittaessa mahdollisia uusia bakteereita kliinisiin kokeisiin. Lisäksi tutkimus osaltaan paljastaa *S. pyogenes* -infektioiden taustalla olevia immunologisia mekanismeja, joita voidaan hyödyntää kehitettäessä menetelmiä hallita bakteerin aiheuttamia voimakkaita immuunivasteita.

Avainsanat: sytokiini, dendriittisolu, makrofagi, luontainen immuunijärjestelmä, bakteeri

Sammandrag

Sinikka Latvala. Activation of Innate Immune Responses by Non-pathogenic and Pathogenic Bacteria in Human Leukocytes [Aktivering av leukocyter i det naturliga immunförsvaret orsakad av icke-pathogena och pathogena bacterier] Institutet för hälsa och välfärd (THL), Forskning 138. 86 sidor. Helsinki, Finland 2014. ISBN 978-952-302-312-3 (tryckt); ISBN 978-952-302-313-0 (nätpublikation)

Människans immunförsvar och epitelceller är i ständig växelverkan med mikrober. En del av mikroberna är oförargliga, eller till och med nyttiga, medan andra kan orsaka sjukdom. Immunförsvaret kan delas in i det naturliga och det adaptiva immunförsvaret. Det naturliga immunförsvaret bekämpar skadliga mikrober med hjälp av ätarceller som t.ex. makrofager och antigenpresenterande dendritceller. Med hjälp av olika receptorer känner de här cellerna igen ytstrukturer hos sjukdomsalstrande bakterier och producerar rikligt med cytokiner. Typen av cytokiner beror på mikroben eller antigenen som utlöser produktionen samt vilken av kroppens celler mikroben eller antigenen kommer i kontakt med. Cytokinproduktionen är starkt reglerad och felaktig identifiering av mikrober eller antigener kan leda till ett för svagt eller å andra sidan ett för kraftigt immunsvar.

I den här avhandlingen granskades växelverkan mellan människans leukocyter och grampositiva bakterier genom att använda dendritceller och makrofager som differentierats från monocyter isolerade från friska blodgivare. Resultaten visar att det finns klara skillnader mellan hur probioter, potentiella probioter och patogena S*treptococcus pyogenes* -bakterier aktiverar immunsvaret. Vidare bestämdes bakteriernas anti-inflammatoriska potential som kan ha betydelse för deras kliniska verkan.

Dendritceller som stimulerats med bakterier studerades och cellernas utveckling, aktivering och cytokinproduktion jämfördes med det immunsvar som makrofagerna producerade. Syftet var att undersöka om det finns skillnader mellan hur olika bakterier aktiverar cellerna i människans immunförsvar. De icke-patogena bakterierna fick dendritcellerna att mogna och uttrycka co-stimulatoriska molekyler på sin yta men bakteriernas förmåga att aktivera cytokinproduktionen varierade markant. En del av bakterierna aktiverade inte cytokinproduktionen alls medan andra orsakade kraftigare cytokinproduktion än sjukdomsalstraren *Streptococcus pyogenes*. Delvis mogna dendritceller som uttycker co-stimulatoriska molekyler på sin yta men inte producerar cytokiner fungerar sannolikt som en del av det tolerogena immunsvaret och främjar kroppens tolerans för normalflorans mikrober. Alla undersökta bakterier orsakade å andra sidan ett kraftigt eller relativt kraftigt inflammatoriskt svar hos makrofager, vilket tyder på att bakteriens förmåga att

orsaka sjukdom kanske inte har så stor betydelse för det naturliga immunsvar som makrofagerna utvecklar.

I undersökningen användes olika varianter av *S. pyogenes*: en bakteriestam av vildtyp och två bakteriestammar som vardera saknade någon av streptolysin-virulensfaktorerna. När dessa bakteriestammars förmåga att aktivera vita blodceller jämfördes visade resultaten att streptolysinerna inte är nödvändiga för att ge upphov till ett inflammatoriskt svar. Vidare undersöktes den dynaminmedierade endocytosens roll i aktiveringen av immunsvaret.

Resultaten från den här undersökningen hjälper oss att förstå hur det naturliga immunförsvaret fungerar i växelverkan med olika bakterier. Kunskapen kan utnyttjas för att undersöka de hälsoeffekter som probiotiska bakterier ger upphov till och kan hjälpa till att identifiera nya potentiellt gynnsamma bakteriearter för kliniska försök. Resultaten avslöjar också nya detaljer om de immunologiska mekanismer som ligger bakom infektioner orsakade av *S. pyogenes*. Dessa kan utnyttjas för att utveckla metoder som hjälper till att dämpa det kraftiga inflammationssvar som orsakas av bakterien.

Nyckelord: cytokin, dendritcell, makrofag, det naturliga immunförsvaret, bakterie

Contents

List of original publications

I Latvala S, Pietilä TE, Veckman V, Kekkonen RA, Tynkkynen S, Korpela R, Julkunen I. Potentially probiotic bacteria induce efficient maturation but differential cytokine production in human monocyte-derived dendritic cells. World J Gastroenterol. 2008; 14:5570-83.

II Latvala S, Miettinen M, Kekkonen RA, Korpela R, Julkunen I. *Lactobacillus rhamnosus* GG and *Streptococcus thermophilus* induce suppressor of cytokine signalling 3 (SOCS3) gene expression directly and indirectly via interleukin-10 in human primary macrophages. Clin Exp Immunol. 2011; 165:94-103.

III Latvala S, Mäkelä SM, Miettinen M, Charpentier E, Julkunen I. Dynamin inhibition interferes with inflammasome activation and cytokine gene expression in *Streptococcus pyogenes* -infected human macrophages. Clin Exp Immunol. 2014; 178:320-333.

Abbreviations

AIM	absent in melanoma
APC	antigen-presenting cell
ASC	apoptosis-associated speck-like protein
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
CARD	caspase recruitment domain
CD	cluster of differentiation
cDNA	complementary DNA
Ci	Curie
CHX	cycloheximide
CsA	cyclosporin A
Ct	comparative threshold
DAI	DNA-dependent activator of IRFs
DC	dendritic cell
ds	double-stranded
DSM	German collection of microorganisms
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
GAS	group A streptococcus
GM-CSF	granulocyte-macrophage colony stimulating factor
IBD	inflammatory bowel disease
IBS	inflammatory bowel syndrome
IFN	interferon
IκB	inhibitor of nuclear factor kappa-B
IL	interleukin
IRF	interferon regulatory factor
IRAK	IL-1 receptor associated kinase
IU	international unit
JAK	Janus kinase
LGP2	laboratory of genetics and physiology 2 protein
LPS	lipopolysaccharide
LRR	leucine-rich repeat
LTA	lipoteichoic acid
MAMP	microbe-associated molecular pattern
MAPK	mitogen-activated protein kinase
MDA	melanoma differentiation-associated gene
mDC	myeloid DC
MDP	muramyl dipeptide
MHC	major histocompatibility complex
moDC	monocyte-derived dendritic cell
MOI	multiplicity of infection
mRNA	messenger RNA

MRS	de Man, Rogosa and Sharpe medium
MyD88	myeloid differentiation primary response gene 88
NAIP	neuronal apoptosis inhibitory protein
NALP	NACHT, LRR and PYD-domain containing protein
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor kappa-B
NK	natural killer
NLR	nucleotide-binding domain and LRR containing gene family
NLRC	NLR family, CARD domain containing
NOD	nucleotide-binding oligomerization domain
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
pDC	plasmacytoid dendritic cell
PDTC	pyrrolidine dithiocarbamate
pfu	plaque-forming unit
PG	peptidoglycan
PKR	protein kinase R
PRR	pattern recognition receptor
RIG-I	retinoic acid-inducible gene I
RLR	RIG-I-like receptor
SARM	sterile alpha and HEAT/Armadillo motif protein
SDS	sodium dodecyl sulphate
SLE	systemic lupus erythematosus
SLO	streptolysin O
SLS	streptolysin S
SOCS	suppressor of cytokine signalling
ss	single-stranded
STAT	signal transducer and activator of transcription kinase
TGF	transforming growth factor
Th	T helper cell
TIR	Toll/IL-1 receptor
TIRAP	TIR domain containing adaptor protein
TLR	Toll-like receptor
TNF	tumor necrosis factor
TRAM	TRIF-related adaptor molecule
TRAF	TNF receptor-associated factor
Treg	regulatory T cell
TRIF	TIR domain-containing adaptor protein inducing IFN-β

1 Introduction

The human body is continuously exposed to a variety of microorganisms most of which are beneficial or harmless to the host. Mechanical and chemical barriers that separate the external environment from the internal sterile milieu protect the host from pathogenic microorganisms that occasionally cause severe infections. Mechanical barriers such as the epidermis and mucosa that line the internal body cavities effectively prevent pathogen entry when intact. Additionally, chemical barriers such as low pH and secreted antimicrobial compounds make the milieu unfavorable for bacterial growth. Sometimes these barriers are breached by an injury or via animal vectors allowing pathogenic bacteria to cross the epithelium, sometimes leading to an infectious disease. During the course of evolution, non-pathogenic bacteria of the normal microbiota have developed commensal and mutually beneficial relationships with their hosts, and pathogenic bacteria have evolved several strategies to enter the host and avoid host immune responses.

The intestine is a potential entry route for a number of pathogenic bacteria. The integrity of the epithelial barrier in the intestine can be improved by bacteria with health promoting properties termed probiotics. They act through diverse mechanisms to maintain intestinal homeostasis and prevent pathogen growth and attachment to the mucosal and epithelial surfaces. In addition, the ability of probiotic bacteria to modulate the intestinal immune responses has been demonstrated.

The cellular components of the immune system that detect and eliminate the invaders are divided into two arms; innate and adaptive responses. Innate immunity represents the front line of immune responses against invading pathogens. It provides unspecific recognition of microorganisms by complement, destruction of infected cells by natural killer (NK) cells, and rapid activation of phagocytic cells including macrophages, dendritic cells (DC), and neutrophils that detect, ingest, and destroy foreign material. Innate immunity also includes soluble molecules that enhance antimicrobial resistance or are directly involved in bacterial destruction such as defensins. Cells of the innate immune system are scattered throughout the body to act as sentinels of danger signals. Recognition of microbes is based on the germ-line encoded pattern recognition receptors (PRRs) that sense conserved microbial structures absent from the host. In response to microbial stimuli, these cells secrete soluble mediators such as cytokines and chemokines to initiate appropriate immune responses.

When the innate immune system is unable to clear the infection, the adaptive arm of the immune system is activated. It complements the innate immune system and is responsible for the elimination of pathogens at later phase of infection. DCs act in the interface of innate and adaptive immune system to activate T and B cell functions. This highly specific part of the immune system acts through clonally selected receptors. Adaptive immunity is activated slowly, in days or weeks, and it is responsible for the immunological memory and protective immunity. Upon reinfection, the immunological memory offers rapid and efficient responses against pathogens. Adaptive immunity also contributes to the development of autoimmune diseases, allergy, and allograft rejection.

Innate and adaptive parts of the immune system communicate through cytokines, produced by antigen presenting cells (APCs), macrophages and DCs. Secretion of cytokines in response to microbial stimulation must be tightly regulated to avoid tissue destruction and detrimental effects on the host. On the other hand, the ability of non-pathogenic and probiotic bacteria to induce host cell cytokine production, might have beneficial immunomodulatory effects. The ability of non-pathogenic bacteria to fine tune the inflammatory responses offers an intriguing field for research with a great potential to modulate host immune responses.

In the present work the cytokine profiles induced by non-pathogenic bacteria and their abilities to activate DC maturation were analysed and compared with the reponses induced by a pathogenic bacterium *Streptococcus pyogenes* (group A streptococcus, GAS). The immune responses of multiple bacterial strains were compared in human monocyte-derived (mo)DC and macrophage cell models. Moreover, we addressed the question, how non-pathogenic bacteria-induced responses are regulated and what are the key molecules controlling these actions. In addition to non-pathogenic bacteria-induced responses, the role of bacterial virulence factors called streptolysins of pathogenic GAS and the role of dynamin-dependent endocytosis in GAS-induced inflammatory responses were analysed in the macrophage model to obtain better understanding of their importance and contribution to GAS infections.

2 Review of the literature

2.1 Phagocytic cells of the immune system

One of the mechanisms to control pathogenic invasion is the ability of immune cells to internalize microbes from their milieu in a process called phagocytosis. Professional phagocytes such as neutrophils, macrophages, and DCs utilize phagocytic receptors to take up also other foreign particles and apoptotic cells. DCs and macrophages are distinguished from other phagocytes by their ability to act as APCs and to initiate adaptive immune responses via T and B cell activation (Figure 1).

2.1.1 Macrophages

Macrophages are derived from myeloid progenitor cells in the bone marrow. These precursor cells develop into monocytes and circulate in blood before homing into different tissues where they give rise to a variety of tissue resident macrophages or specialized cells such as dendritic cells [1-3]. Macrophages are found throughout the body and they show great functional diversity by participating in different physiological and pathological processes including development, bone remodeling, wound healing, and removal of apoptotic cells [4]. Macrophages are involved in the elimination of pathogens by detecting, ingesting, and destroying them and they can also act as APCs to initiate adaptive immune responses. Tissue macrophages are replaced by newly recruited blood monocytes as they undergo programmed cell death within weeks or months [5]. However, also local proliferation of tissue macrophages has been suggested [6].

The basal activity as well as the ability of macrophages to respond to inflammatory stimuli varies considerably depending on their location and cytokine milieu of their microenvironment [3, 4, 7]. Macrophage activation and differentiation are also dependent on their cell surface receptors or intracellular molecules, which mediate phagocytosis and activation of signalling cascades, respectively. These receptors include complement, scavenger, and Fc-receptors as well as lectin receptors, cluster of differentiation (CD) 14, and Toll-like receptors (TLRs) [4, 8].

Homeostatic processes are mediated independently of immune cell signalling, but in response to inflammatory stimuli macrophages activate multiple signalling pathways

resulting in the production of various inflammatory mediators including cytokines and chemokines. The differentiation lineage of macrophages depends on the signals they receive from the environment. The microbicidal activity of macrophages is enhanced by cytokines, including interferon (IFN)-γ, produced by other immune cells such as NK and T cells. Instead, regulatory macrophages are generated in response to various stimuli including immune complexes, prostaglandins, and interleukin (IL)-10. These regulatory cells suppress immune responses via increased IL-10 production. In addition, the cytokines produced by macrophages can influence their own activation in an autocrine fashion [3, 4]. Since excessive cytokine production can lead to tissue damage of the host, the activation of macrophages must be tightly controlled. Nevertheless, macrophages are shown to contribute to the development of allergy and hypersensitivity and they play a pathogenic role in several autoimmune diseases [3, 4].

Macrophages differ morphologically and phenotypically in different tissues. The largest reservoir of macrophages resides in the gastrointestinal mucosa, which is the largest body surface to interact with the external environment [5]. The first cells to contact microbes after the epithelial barrier are APCs and intraepithelial lymphocytes. Intestinal macrophages have a unique phenotype characterized by strong phagocytic and bactericidal activity with downregulated proinflammatory functions. They are recruited to lamina propria by endogenous chemoattractants in the non-inflamed mucosa and by bacterial products and inflammatory chemokines during inflammation. Interestingly, resident intestinal macrophages do not mediate mucosal inflammation, since their proinflammatory cytokine production is downregulated, regardless of whether the stimulus is a soluble factor or a phagocytic event [9, 10]. It is believed that the newly recruited blood monocytes are involved in the inflammatory reactions in the gut [5].

Some pathogenic bacteria such as *Listeria*, *Salmonella*, *Shigella*, and *Legionella* have evolved strategies to survive inside macrophages by interfering with the molecules regulating their recognition or destruction by phagocytes [11-13].

2.1.2 Dendritic cells

DCs are the most potent APCs that initiate and control the quality of adaptive immune responses (Figure 1). DCs reside in an immature state in peripheral tissues near potential pathogen entry sites such as the skin, mucosal surfaces, and lymphoid organs. As immature DCs they continuously monitor their milieu in the tissues and take up particles and soluble components. DCs are involved in bacterial recognition, induction of tolerance, and shaping of T cell-mediated responses. DCs are able to

present their antigens to naïve T cells, whereas macrophages only activate primed T cells [14].

DCs can arise from myeloid or lymphoid progenitors. DC subsets representing the myeloid DC (mDC) lineage can be found in peripheral tissues, secondary lymphoid organs, and in blood. Another DC subset, plasmacytoid DCs (pDC) of lymphoid origin, circulate in blood [15]. DC subsets can be classified based on their surface molecules of myeloid or lymphoid lineage or their location as they express different TLRs and produce a distinct sets of cytokines in response to microbial stimulation [15, 16].

Upon encountering an antigen, DCs undergo a maturation process including morphological changes, reduction in endocytic capacity, and secretion of cytokines and chemokines. Maturation also includes increased expression of major histocompatibility complex (MHC) molecules and costimulatory molecules such as CD80 and CD86 required for T cell activation. During maturation DCs migrate into the peripheral lymph nodes to present peptides from ingested antigens to T and B cells to initiate adaptive immune responses [14]. The outcome of the immune response is influenced by the extracellular environment, nature of the signal, and the type of DCs responding to it [17].

Depending on the stimulus, DCs produce different cytokines to induce T cell polarization. IL-12 family cytokines support Th1 polarization which is important in protection against intracellular pathogens and Th2 polarization induced by IL-4 is important against extracellular and parasitic pathogens. While IL-10 favors the generation of Treg cells that suppress inflammatory reactions and are critical in maintaining tolerance, IL-1β and IL-23 support Th17 differentiation which is involved for example in intestinal immune responses and in autoimmune inflammation [18-20]. However, DCs show considerable plasticity in their functions and can change their polarization in response to alterations in their local cytokine milieu [21].

DCs have been shown to participate in unwanted and uncontrolled immune responses including allergy, autoimmunity, cancer, and transplant rejection [15, 22]. Furthermore, DCs are targets for microbes that have evolved strategies for disturbing DC functions and their recognition and degradation by immune cells. Microbes can induce apoptosis and interfere with DC maturation and migration to prevent the initiation of protective immune responses. Some pathogenic bacteria use DC receptors to enter the cells and modulate the cytokine profiles produced by the cells in favour for bacterial survival [15, 23].

In the gastrointestinal tract DCs are an important cell type responding to microbial entry into the underlying tissues. The recognition of bacteria as either commensal or pathogenic and whether to activate an inflammatory, tolerizing, or no response is an important challenge to the mucosal immune system. It has been reported that DCs residing under the epithelial cells in lamina propria, can reach their dendrites through the tight junctions of epithelial cells into the gut lumen and sample for antigens or microbes [24, 25].

A challenge in human DC studies has been the low number of mDCs and pDCs in human blood. Most of the *in vitro* studies utilizing human DCs have been conducted with monocytes that are differentiated into DCs in the presence of IL-4 and granulocyte-macrophage colony stimulating factor (GM-CSF) [26]. These cells are called monocyte-derived DCs (moDCs) and they closely resemble blood-derived mDCs [27, 28].

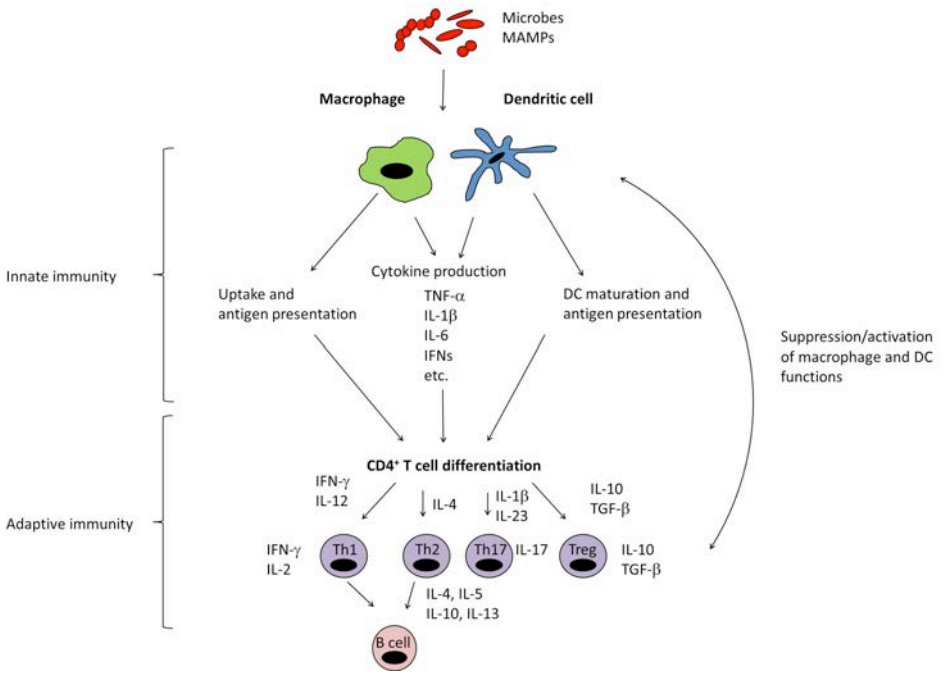

Figure 1. Schematic representation of the interplay between the cellular innate and adaptive immune responses. Interactions between macrophages, DCs, and $CD4^+$ T cells are shown.

2.2 Microbe recognition

Rapid recognition of pathogens and other danger signals is necessary for the activation of immune responses. Cells of the innate immune system, including macrophages and DCs recognize conserved microbe-associated molecular patterns (MAMPs) and intracellular danger signals through different kinds of receptors (Table 1). These receptors include TLRs, nucleotide-binding and leucine-rich repeat containing gene family receptors (NLRs), and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) which are able to activate intracellular signalling pathways resulting in the expression of genes that contribute to the magnitude and quality of immune responses.

2.2.1 Toll-like receptors (TLRs)

TLRs are critical components of the innate immune system recognizing both microbe-specific molecules and damaged host cell components [29-31]. The Toll protein was initially discovered in *Drosophila* [32]. Currently, 13 members of the TLR family have been identified in mammals, of which 10 are found in humans. These type I transmembrane glycoproteins consist of a leucine-rich repeat (LRR) domain responsible for ligand recognition and receptor dimerization, and a cytoplasmic Toll/IL-1 receptor (TIR) domain that controls signalling. Ligand engagement by TLR leads to receptor dimerization and activation of intracellular signalling pathways including adaptor molecules, kinases, and transcription factors ultimately leading to the transcription of the genes responsible for immune responses [33, 34]. The most important cell types that express TLRs are macrophages, DCs, and B lymphocytes. Also other cell types express these receptors constitutively or upon microbial stimulation [35, 36].

TLRs can be located on the cell surface (TLRs 1, 2, 4, 5, 6, and 10) or in intracellular compartments such as endosomes and lysosomes (TLRs 3, 7, 8, 9) (Figure 2). For most TLRs ligand specificity has been identified. TLR2 associates with TLR1 or TLR6 to recognize Gram-positive bacterial cell wall components including lipoproteins, lipoteichoic acids (LTA), and peptidoglycan (PG). TLR4 and TLR5 in turn recognize lipopolysaccharide (LPS) from Gram-negative bacteria and the structural epitope of bacterial flagellin, respectively. TLRs 3, 7, 8, and 9 respond to microbe-derived nucleic acids, such as single-stranded or double-stranded RNA and DNA or unmethylated CpG motifs of bacterial DNA [34]. Localization and ligand for TLR10 are still unclear, however, bacterial, viral, and fungal ligands and association with TLR2 on the cell surface have been suggested [37, 38]. The TLR system is very complex since one microbe can activate several TLRs via different

MAMPs to trigger multiple downstream signalling pathways for rapid inflammatory responses. The expression levels of TLRs and the distribution of these receptors on different cell types reflects their ability to respond to specific stimuli. This determines in part the characteristic patterns of cytokines, chemokines, and interferons that are induced by different microbes in various cell types.

Traditionally, Gram-positive bacteria are considered to be recognized by TLR2, one of the most extensively studied receptors in innate immunity. Heterodimerization of TLR2 with TLR1 or TLR6, and possibly with TLR10, gives the receptor the ability to recognize ligands with diverse structures such as LTA, lipoproteins, PG, porins, and virus capsids [39, 40]. Both TLR2/TLR1 and TLR2/TLR6 receptor dimers activate the same signalling cascade, resulting in the activation of identical genes [41]. TLR2 heterodimers are known to associate with co-receptors and accessory molecules such as CD36 [42], CD14 [43, 44], and Dectin-1 [45] to expand the ligand repertoire.

2.2.2 Activation of signalling pathways via TLRs

A number of different signalling pathways are activated in response to ligand recognition by TLRs. The cytoplasmic TIR domains of TLRs are responsible for downstream signalling. These domains recruit TIR domain containing adaptor molecules, including myeloid differentiation primary response gene 88 (MyD88) protein, TIR domain containing adaptor protein (TIRAP), TIR domain containing adaptor inducing IFN-β (TRIF), TRIF related adaptor molecule (TRAM), and sterile alpha and HEAT-Armadillo motifs (SARM) to transmit downstream signalling. Three major signalling pathways mediate TLR-induced responses including nuclear factor kappa-B (NF-κB), mitogen activated protein kinases (MAPKs), and IFN regulatory factors (IRFs). Activation of these downstream signalling pathways culminates in cellular responses including cytokine and chemokine production, maturation of APCs, and initiation of innate and adaptive immune responses (Figure 2) [33, 46, 47].

TLR-induced signalling pathways can be largely divided into MyD88-dependent and -independent pathways. The adaptor protein MyD88 is involved in the signalling triggered by all TLRs except for TLR3. MyD88-dependent signalling through TLRs 2, 4, and 5 primarily result in MAPK and NF-κB-dependent proinflammatory responses. Moreover, MyD88-dependent type I IFN expression occurs through activation of intracellular TLR7, 8, and 9. The MyD88-independent pathways downstream of TLR3 and TLR4 signal through TRIF to activate IRFs which ultimately lead to type I IFN production [48, 49]. Type I IFN production is

traditionally associated with the recognition of nucleic acids of viral or bacterial origin by TLR3, 7, 8, or 9 or through internalized TLR4 via TRIF activation [48]. Recently, type I IFN production after TLR2 activation by whole bacteria or bacterial components [50, 51] was suggested to occur in endosomes after TLR2 internalization [52, 53].

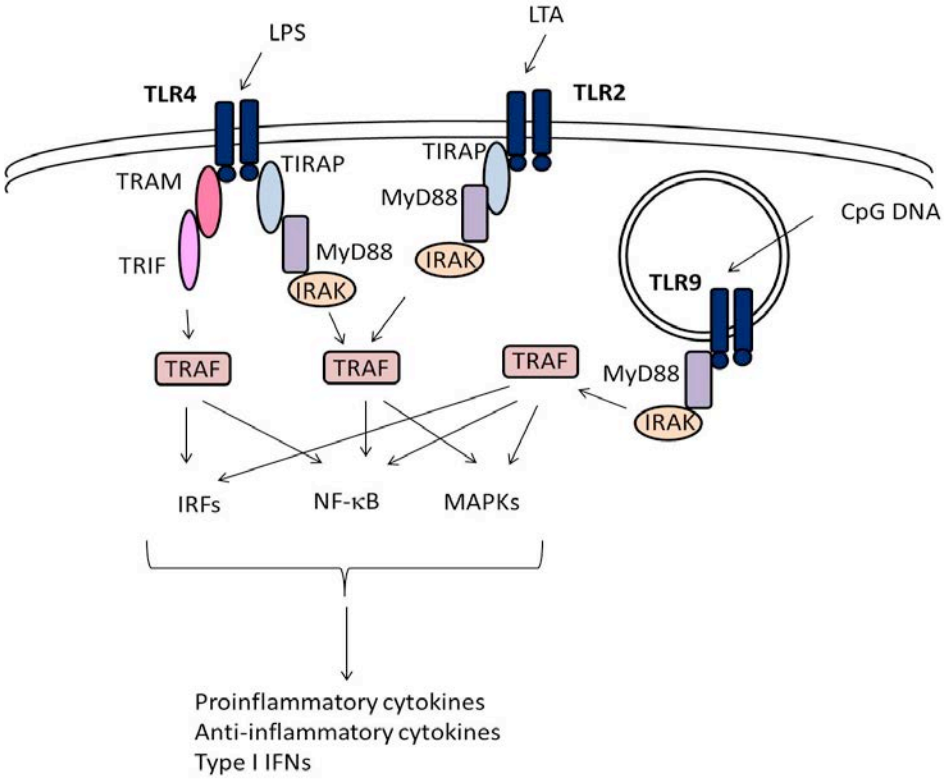

Figure 2. **Overview of the signal transduction via TLRs.** Representative receptors for Gram-negative (TLR4) and Gram-positive (TLR2) bacterial ligands as well as for microbial nucleic acids (TLR9) are presented. TLRs can be expressed on the cell surface (TLR4, TLR2) or in intracellular compartments such as endosomes (TLR9). Ligand binding triggers recruitment of adaptor molecules and initiates a complex signal transduction pathway ultimately leading to the expression of cytokine genes. MyD88 is involved in all TLR signalling (except TLR3). It couples IL-1 receptor associated kinases (IRAKs) and transforming growth factor β-associated kinases (TRAFs) for downstream signalling. Ligand binding to TLR4 can also trigger MyD88-independent signalling pathways via TIR domain-containing adaptor inducing IFN-β (TRIF) and TRIF related adaptor molecule (TRAM). Depending on the receptor, MAPK, NF-κB, and IRF pathways can be activated.

Table 1. Overview of human receptors of the innate immune system and their most common bacteria/virus –derived MAMPs.

Receptor family	PRR	MAMP	Origin of the ligand	Cellular localization
TLR	TLR1-TLR2	triacyl lipopeptides	bacteria, mycobacteria	cell surface
	TLR2-TLR6	diacyl lipopeptides	mycobacteria	cell surface
		lipoteichoic acid	bacteria	
	TLR2	peptidoglycan	bacteria	cell surface
		lipoarabinomannan	mycobacteria	
	TLR3	dsRNA	RNA viruses	endosomes
				cell surface
	TLR4	lipopolysaccharide	bacteria	cell surface
		envelope proteins	viruses	
	TLR5	flagellin	bacteria	cell surface
	TLR7	ssRNA	RNA viruses	endosome
		RNA	bacteria	
	TLR8	ssRNA	RNA viruses	endosome
	TLR9	CpG DNA	bacteria	endosome
		DNA	viruses	
	TLR10	unknown	bacteria	cell surface
			viruses	
RLR	LGP2	RNA	viruses	cytosol
	MDA-5	RNA	viruses	cytosol
	RIG-I	RNA	bacteria	cytosol
			viruses	
NLR	NAIP5	flagellin	bacteria	cytosol
	NLRC4	flagellin	bacteria	cytosol
	NALP1	muramyl dipeptide	bacteria	cytosol
		anthrax lethal toxin		
	NALP3	muramyl dipeptide	bacteria	cytosol
		RNA	bacteria, viruses	
		toxins	bacteria	
	NOD1	diaminopimelic acid	bacteria	cytosol
	NOD2	muramyl dipeptide	bacteria	cytosol
		ssRNA	viruses	
Others	AIM2	dsDNA	bacteria, viruses	cytosol
	DAI	dsDNA	bacteria, viruses	cytosol
	PKR	dsRNA	viruses	cytosol

Abbreviations: **AIM2**, absent in melanoma 2; **DAI**, DNA-dependent activator of IRFs**; LGP2**, laboratory of genetics and physiology 2; **MAMP**, microbe-associated molecular pattern; **MDA5**, melanoma differentiation-associated gene 5; **NAIP5**, neuronal apoptosis inhibitory protein 5; **NALP**, NACHT, LRR and PYD-domain containing protein; **NOD**, nucleotide-binding oligomerization domain; **NLR**, nucleotide-binding and leucine-rich repeat containing gene family receptor; **NLRC**, NLR family, CARD domain containing; **PKR**, protein kinase R; **PRR**, pattern recognition receptor; **RIG-I**, retinoic acid-inducible gene I; **RLR**, RIG-I-like receptor; **TLR**, Toll-like receptor. Adapted from: [34, 37, 38, 49, 54-56].

2.2.3 Cytoplasmic surveillance of microbes and host danger signals

TLR-independent recognition of pathogens is accomplished by cytosolic PRRs including NLRs and RLRs. RLRs sense cytosolic RNA which results in type I IFN mediated antiviral responses. Other cytoplasmic receptors such as DAI and AIM2 contribute to inflammatory responses by recognizing cytosolic DNA leading to type I IFN production and proinflammatory responses (Table 1) [49].

NLRs consist of centrally located nucleotide-binding oligomerization (NOD), C-terminal leucine-rich repeat (LRR), and N-terminal caspase recruitment (CARD) domains which mediate oligomerization, ligand sensing, and signal initiation, respectively [49]. Most of the NLR family members fall into two large subclasses; NODs and NALPs (NACHT, LRR, and pyrin-domain-containing proteins). The best-characterized NLRs are NOD1 and NOD2 which recognize for example bacterial peptidoglycan-derived components from Gram-negative and Gram-positive bacteria [57]. In addition to the activation of proinflammatory gene expression via NF-κB and MAPK activation, NOD proteins can also induce type I IFN signalling in response to bacteria [58].

Dysfunction of NOD proteins is associated with asthma, atopic eczema, and increased incidence of Crohn's disease [59-61]. The increased Th1 responses in Crohn's disease are probably due to disturbed immune responses to bacteria in the intestinal tract such as dysregulated NF-κB activation and Th1 polarization and decreased production of antimicrobial peptides [62, 63].

Other NLR proteins are components of large intracellular complexes, referred to as the inflammasomes that are important in the induction of host defence especially against bacterial infections. Inflammasomes act as platforms for the activation of caspase-1, a proteolytic enzyme responsible for the processing of IL-1β and IL-18 precursors into their biologically active forms (Figure 3) [64, 65]. The best-characterized inflammasomes are NLRC4, NALP3, and AIM2. Typical inflammasomes are composed of the adaptor ASC (apoptosis-associated speck-like protein containing CARD), pro-caspase-1, and NLR family member such as NLRC or NALP. Oligomerization through CARD-CARD interactions results in the activation of caspase-1. The inflammasome activation in macrophages requires two steps: the activation of NF-κB via TLRs to produce pro-IL-1β and pro-IL-18, and the activation of NALP and caspase-1 to cleave these inactive cytokines into their mature forms [66]. Each member of the inflammasome family recognizes different MAMPs through their respective NLR.

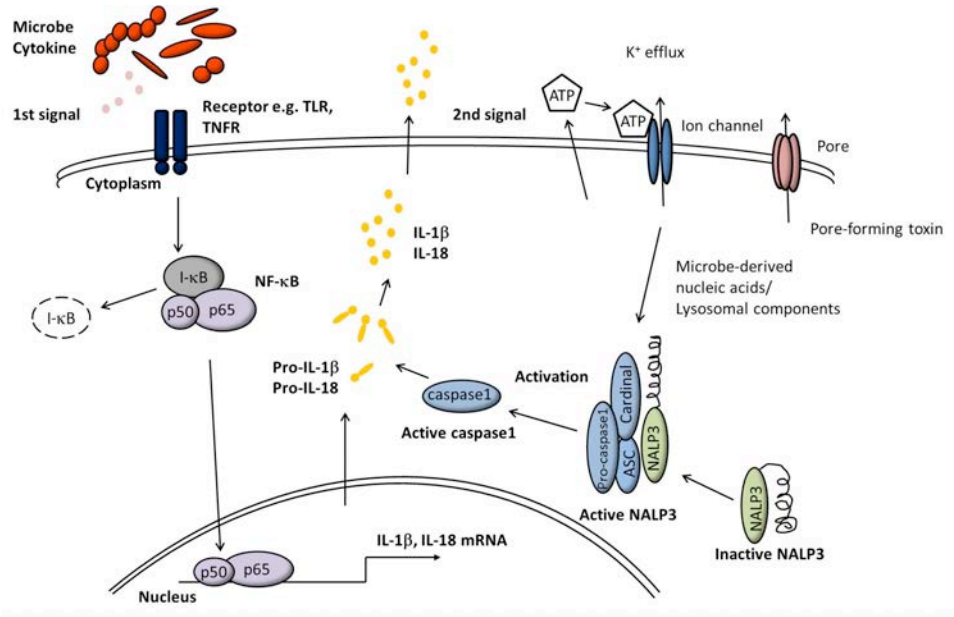

Figure 3. **Schematic representation of NALP3 inflammasome activation.** The activation of NALP3 inflammasome leading to caspase-1 activation and cleavage of pro-IL-1β and pro-IL-18 into their active forms is a two-step process. Signal 1 is needed for NF-κB activation and expression of NALP3, pro-IL-1β, and pro-IL-18. The first signal can be provided by microbial compounds or cytokines such as TNF-α. Signal 2 leads to the activation of NALP3, recruitment of adaptor proteins ASC and Cardinal, and activation of pro-caspase-1 into its active form. Activated caspase-1 cleaves the immature cytokines into their biologically active forms, IL-1β and IL-18. The second signal may result from the release of lysosomal compounds into the cytosol or from the decrease in intracellular K^+ either through pore-forming toxins from bacteria or by the engagement of extracellular ATP with P2X7 receptor. Adapted from [65].

NALP3 is upregulated in cells after TLR stimulation and it is activated in response to a variety of MAMPs and danger signals. It has been suggested that changes in intracellular ion concentrations induce inflammasome activation. The NALP3 inflammasome is activated in response to ATP and previous reports show that priming of cells with LPS is necessary for ATP-induced inflammasome activation [67, 68]. It has been suggested that extracellular ATP with subsequent K^+ efflux via P2X7 receptor triggers pore formation by pannexin 1 ultimately leading to the delivery of microbial products into the cytoplasm to activate the inflammasome [57].

Additionally, some bacterial pore forming toxins [69], reactive oxygen species [70], uric acid crystals [71], and the release of lysosomal compounds such as degradation products of phagocytosed bacteria into the cytosol can trigger inflammasome activation [72]. In addition, pathways other than caspase-1 activation have been proposed to regulate IL-1β and IL-18 processing [73-76].

TLRs and NLRs induce synergistic and simultaneous innate inflammatory responses due to shared ligands, distinct cellular locations, and independent signalling pathways. It is known that NALP1 interacts with NOD2 to mediate caspase-1 activation in response to muramyl dipeptide (MDP) recognition. Thus, suggesting that also NOD2 has a role in both pro-IL-1β synthesis and maturation [49].

2.2.4 Uptake of microbes by phagocytosis and endocytosis

Internalization of foreign particles or microbes into cells is divided into phagocytosis, macropinocytosis, and endocytosis (Figure 4). Macropinocytosis and phagocytosis are actin-dependent internalization mechanisms used in the uptake of fluids and large particles such as bacteria and dead cells. Phagocytosis is restricted to phagocytic cells such as macrophages, DCs, monocytes, and neutrophils. These cells possess phagocytic receptors, such as dectin 1, Fc, and complement receptors, which enable them to recognize targets either directly or indirectly via opsonization. Recognition is followed by the internalization of the microorganism into a phagosome, which then fuses with endosomes and ultimately lysosomes resulting in the degradation of the pathogen. Additionally, phagosomes can be formed inside cells during the engulfment of cellular organelles, cell debris, or intracellular microorganisms. This process is called autophagy. Phagocytosis forms a platform for TLR activation and is in many cases necessary for the activation of cytosolic PRRs and canonical and non-canonical inflammasomes. However, inflammatory responses such as cytokine production do not necessarily require internalization of the target [77-80].

Endocytosis is an important mechanism controlling the composition of the plasma membrane and the responses of cells to their environment in all cell types except in red blood cells. It is involved in many cellular events including changes in cell shape, nutrient uptake, and cell signalling. Several different types of endocytosis have been described based on the molecules that drive the process, including clathrin-dependent, caveolin-dependent, and clathrin- and caveolin-independent endocytosis [81]. Extracellular cargos usually enter cells by several different endocytic pathways. Some internalization pathways are constitutively active, while others are triggered

by specific signals or exploited by pathogens to gain access into subcellular compartments.

Many, but not all endocytic pathways require dynamin, a large GTPase, for pinching and release of vesicles from the plasma membrane (Figure 4) [81, 82]. Dynamin is necessary for many clathrin- and caveolin-dependent pathways and for vesicle formation from various intracellular organelles. Caveolin-mediated endocytosis is involved in delivering lipids, proteins, and pathogens into cells. Dynamin is also involved in some clathrin-independent pathways, such as internalization of cytokine receptor chains [83, 84]. Dynamin 1 and dynamin 2, the best-studied mammalian isoforms, interact with actin binding proteins and influence actin-comet formation on macropinosomes [85].

Invasive microorganisms have evolved several strategies for mediating their own uptake into the cells. Bacteria such as *Listeria monocytogenes* and *Salmonella* can induce their own uptake with the aid of bacterial virulence factors [82,83]. They also have mechanisms to escape from the host detection system, interfere with phagosome maturation, and to avoid phagosomal degradation by escaping into the cytosol.

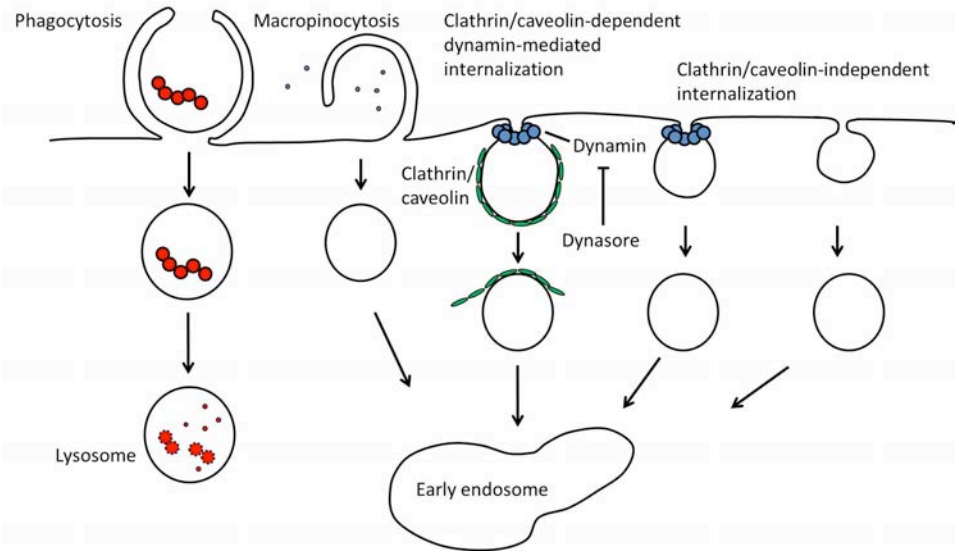

Figure 4. Schematic overview of the entry pathways into cells. Cells take up large particles by phagocytosis and fluids by macropinocytosis, both of which are actin-mediated processes. Cargoes can also be internalized via dynamin-dependent and –independent pathways to be delivered to early endosomes. Dynamin is involved in clathrin and caveolin-dependent and -independent internalization mechanisms by mediating the pinching and release of the vesicle from the plasma membrane. The actions of dynamin can be inhibited with dynasore. Adapted from [81].

2.3 Cytokines

Cytokines are soluble regulatory proteins that mediate the communication between cells to further modulate the innate and adaptive immune responses. Cytokines are secreted by various cell types and can act in autocrine or paracrine fashion to maintain homeostasis, control cell growth and differentiation, and to regulate the quality and magnitude of immune responses. Cytokines act through their specific cell surface receptors to activate intracellular signalling pathways resulting in gene expression of molecules that mediate immune responses. The cytokine system has two typical features, referred to as pleiotropy and redundancy. Thus, cytokines can target several cell types with cell type-specific effects and structurally unrelated cytokines can have similar and overlapping effects on target cells, respectively.

Under natural conditions, cells are exposed to a cocktail of several cytokines and other active components having synergistic and antagonistic interactions, meaning that the exposure of cells to multiple agents at the same time may lead to qualitatively different responses. The production of cytokines must be tightly regulated with a network of cytokine interactions with positive and negative feedback loops, since imbalance in the magnitude and duration of the immune response can be harmful or even lethal to the host. In cytokine production both transcriptional and translational regulation are essential. Transcription of most cytokine genes is regulated by multiple transcription factors activated by diverse ligands. In addition, the stability of cytokine mRNA is tightly regulated by degradation signals. Some cytokines, including IL-1β and IL-18, require posttranslational processing to be functionally mature. Moreover, the secretion of mature cytokines can also be a regulated process [86].

Cytokines are classified based on their structural and functional similarities to interleukin (IL), interferon (IFN), tumor necrosis factor (TNF), colony stimulating factor, transforming growth factor (TGF)-β, chemokine, and growth factor families. Classical proinflammatory cytokines including IL-1β, IL-6, and TNF-α are rapidly produced in response to microbial stimulation to enhance inflammatory reponses. Anti-inflammatory IL-10, on the contrary has immunosuppressive effects. Cytokines with chemotactic effects, chemokines, attract other effector cells to the site of inflammation and direct T cell responses [86]. Additionally, cytokines secreted by APCs direct the generation of Th responses towards Th1, Th2, Th17, or Treg responses. Thus, cytokines produced by various types of immune cells bridge innate and adaptive immunity to efficiently combat microbial infections and maintain tolerance. The complexity of the cytokine network reflects their ability to regulate the functions of different cell populations.

2.3.1 Cytokines inducing Th1 immunity

In response to microbial stimulation or infection both the innate and adaptive arms of the immune response become activated. Cytokines play a significant role in orchestrating both early and late host responses by inducing appropriate T cell polarization with microbial specificity. Polarized T cells modulate the immune response by producing cytokines that function as differentiation and growth factors for Th cells themselves and for other cell types. Th1 cytokines that strengthen cell-mediated immune responses include IL-2, IL-15, IL-18, IL-21, IL-12 family cytokines, and IFNs. Eradication of most intracellular bacteria is regulated by IL-12, IL-18, and IFN cytokine families secreted by differentiated Th1 cells [87].

IFN-γ (Type II IFN)

Probably the most important Th1 cytokine is the type II IFN, IFN-γ, which is mainly produced by NK cells, T cells, and to some extent by macrophages and DCs. IFN-γ is an important activator of macrophages and binds to a receptor composed of IFNγR1 and IFNγR2 subunits expressed on both lymphoid and non-lymphoid cells. The production of IFN-γ is synergistic with other Th1 cytokines and its production is stimulated by APC-T cell interactions. In macrophages and DCs the production of IFN-γ creates an autoregulatory loop that activates both cell types, primes DCs for efficient APC functions, and increases both IFN-γ and IL-12 production. This in turn leads to Th differentiation and adaptive immune responses where IFN-γ can amplify the induction of type I IFNs (IFN-α and IFN-β) to initiate innate and adaptive immune responses [87, 88].

The IL-12 family

IL-12 is a heterodimeric cytokine composed of two subunits p35 and p40. The major cell types producing IL-12 are APCs such as macrophages and DCs [89, 90]. The IL-12 receptor consists of two chains, IL-12Rβ1 and IL-12Rβ2, and it is mostly present on the cell surface of NK cells and activated T cells, although there is some expression in macrophages and DCs. IL-12 links innate immunity to adaptive immunity by inducing the production of IFN-γ and favouring the differentiation of Th1 cells. It is unclear whether IL-12 is required for the initiation of Th1 responses. It may be more critical in inducing IFN-γ production at the later stages of infection [89].

IL-12p40 is produced in large excess over the IL-12p35 subunit and it is known to associate with p19 subunit to form another IL-12 family member, IL-23 [91]. IL-23 binds to a receptor complex composed of IL-12Rβ1 and IL-23R chains. Similar to IL-12, IL-23 is primarily produced by phagocytes in response to microbial stimulation. The main target cells of IL-23 are NK cells and T cells but its receptors are also expressed at low levels on monocytes, macrophages, and DCs [87, 90, 92]. However, in contrast to IL-12, which is important in inducing Th1 responses, IL-23 also plays a role in the survival and maintenance of Th17 cells. These cells produce IL-17 and induce the production of proinflammatory cytokines which can play a role in the development of autoimmunity or protective responses during infection. Th17 cells improve mucosal barrier function and mediate protective mucosal host defense against microbes [18, 92].

The third IL-12 family member IL-27, composed of EBI3 and p28 subunits [93], has a proinflammatory and a limiting role in the intensity and duration of innate and adaptive immune responses [18, 94]. IL-27 is produced primarily by macrophages, and DCs. IL-27 signals through a receptor composed of two subunits; gp130 and a

novel member of the type I cytokine receptor family, IL-27RA (also known as the T cell cytokine receptor TCCR or WSX1) which shows strong homology to the IL-12Rβ2 chain [18, 87]. Elevated IL-27 levels have been associated with Crohn's disease and psoriasis. Additionally, single nucleotide polymorphism in the gene encoding IL-27 may increase the risk for autoimmune diseases such as asthma and inflammatory bowel disease (IBD) [94].

The expression of the subunits of IL-12 family cytokine genes is independently regulated, and the formation of biologically active cytokines requires the synthesis of both subunits within the same cell. All three IL-12 family members mediate immune responses by activating the Janus Kinase (JAK)/Signal transducer and activator of transcription (STAT) pathway with specific differences in the activation and phosphorylation of different STAT molecules [92, 94].

2.3.2 Proinflammatory cytokines

TNF-α

TNF-α is a proinflammatory cytokine expressed especially by activated macrophages and lymphocytes. It contributes to different cellular responses including apoptosis and expression of genes involved in early inflammatory immune responses and adaptive immune responses. TNF-α is expressed as a membrane-bound or secreted ligand that is bioactive as a trimer. Most of the TNF-induced responses are transmitted via the TNFR superfamily members TNFR1 or TNFR2. Depending on the cellular milieu, TNF-α can induce either NF-κB-mediated survival and proinflammatory pathway or an apoptotic response through caspases [95, 96].

IL-1β

IL-1β is a proinflammatory cytokine able to stimulate the expression of genes associated with inflammatory responses. The primary sources of IL-1β are blood monocytes, tissue macrophages, and DCs. IL-1β can be produced as a precursor molecule after bacterial stimulation and it is cleaved into its biologically active form by caspase-1 after inflammasome activation. The most important enzyme responsible for the cleavage of IL-1β is intracellular caspase-1 although, some serine proteinases including elastase, cathepsin G, proteinase 3, and metalloproteinases can also process IL-1β [76, 97]. IL-1β contributes to host defence against infection by activating neutrophils and macrophages to engulf pathogens and by initiating Th1 and Th17 adaptive immune responses. IL-1β also inhibits Th1 polarization by inhibitig IFN-γ production, thus favouring Th17 polarization [97].

2.3.3 Th2 and anti-inflammatory cytokines

The most important Th2 type cytokines, IL-4, IL-5, IL-10, and IL-13, are involved in the generation of antibody-mediated immune responses. These cytokines are produced to eliminate helminths and other extracellular organisms. However, their excessive production is also involved in autoimmune and atopic diseases.

IL-4, IL-10, and TGF-β are anti-inflammatory cytokines that suppress inflammatory reactions by inhibiting the expression of proinflammatory cytokines and attenuating the stimulatory effects of IL-12 and IFN-γ. IL-10 is the most important anti-inflammatory and regulatory cytokine having a crucial role in limiting the immune response to pathogens and preventing the damage to host cells [98-100]. However, also immunostimulatory effects of IL-10 have been observed in some circumstances. IL-10 is expressed by cells of the innate and adaptive immune systems. By acting on T cells, DCs, and macrophages IL-10 has the ability to downregulate cytokine production and inhibit the development of Th1 type immune responses as well as Th2 and allergic responses. IL-10 inhibits the synthesis of several cytokines including IL-1β, IL-6, IL-12, and TNF-α, and downregulates IgE production [101, 102].

2.3.4 Interferons

Interferons are proteins with antiviral activity as well as they have the ability to regulate the functions of innate and adaptive immune cells. Interferons are classified into three classes (type I, type II and type III IFNs) based on the receptor complex they use for signalling. After receptor binding all IFNs activate the JAK/STAT pathway to transduce inflammatory signals. The production and regulation of IFNs involve a positive feedback loop, which can significantly enhance IFN responses [103-105].

Type I IFNs

The major subtypes of type I IFNs are IFN-α and IFN-β. They are produced in response to microbial stimulation leading to the expression of several hundred IFN-stimulated genes (ISGs) [106, 107]. Type I IFNs are produced by almost all cell types and they utilize a receptor that consists of IFNAR1 and IFNAR2 chains for signalling. Especially pDCs produce high amounts of type I IFNs in response to viral stimulation in a MyD88-dependent manner. This may be due to their high expression of TLR7 and 9 [108]. Also macrophages and DCs produce type I IFNs in response to activation of TLR3 and TLR4 pathways via the adaptor protein TRIF [109] and in response to TLR2 activation via MyD88 [50, 52, 110].

In addition to viral stimulation, also bacteria can induce type I IFN production [111-113]. Type I IFNs can regulate the development of adaptive immunity by promoting DC maturation and Th1 polarization [114]. During bacterial infections, type I IFNs can have different functions including a role in antibacterial resistance [106]. IFNs are shown to be involved in host resistance to group B streptococci, pneumococci [115], and *Streptococcus pyogenes* (GAS) [116], but detrimental to host in *Listeria monocytogenes* infection [117, 118].

Type II IFN

The only member of the type II IFN family, IFN-γ is an important Th1 cytokine and is discussed previously in the context of cytokines inducing Th1 immunity.

Type III IFNs

Type III IFNs are the most recently found IFN family members, also known as IFN-λs. This class consists of four homologous genes IFN-λ1-4 [119-121]. IFN-λs signal through a receptor composed of IL10R2 and IFNλR1 chains principally restricted to cells of epithelial origin. Type III IFNs activate the same intracellular signalling pathway as type I IFNs. Thus, they have rather similar biological activities [88].

2.3.5 Chemokines

Cytokines with chemotactic properties are called chemokines. Members of the chemokine superfamily can be grouped into four subfamilies, CC, CXC, CX3C, and XC, based on the arrangement of their N-terminal cysteine residues. Most chemokines belong to CC or CXC classes, where the first two cysteines are either adjacent or separated with one amino acid, respectively. Chemokines can also be classified into inflammatory and homeostatic chemokines although some of them can exhibit both functions. Inflammatory chemokines that mainly attract macrophages and neutrophils create a chemical gradient to guide cells to the site of inflammation. Homeostatic chemokines are produced constitutively in the bone marrow and thymus to regulate leukocyte hematopoiesis and to guide leukocyte migration to the secondary lymphoid organs. Chemokines can be involved in both inflammatory reactions against pathogens and in autoimmunity. Different leukocyte subsets express distinct chemokine receptor patterns on their surface forming the basis for the selective activities of chemokines. To complicate the chemokine network, they show redundancy and binding promiscuosity and some chemokines can act as antagonists for other ligand-receptor pairs [105, 122-125].

Chemokines have an important role in regulating DC trafficking during inflammation. Chemokines including CXCL10 and CCL20 are produced by DCs in

response to microbial stimuli [126, 127]. The binding of CCL20 to its receptor CCR6 on the surface of immature DCs guides these cells to the site of infection. Ligand binding and antigen uptake during infection downregulate CCL20 chemokine receptor expression on DC surface. Thus, decreasing the ability of DCs to respond to inflammatory chemokines. Upon maturation DCs start to express CCR7, a receptor for CCL21 and CCL19 which attract naïve T cells [126-129]. The expression of CCL19 and its receptor by mature DCs lead to DC migration to the local draining lymph nodes and attract naïve T cells into their vicinity to be activated [125, 127, 129, 130].

Table 2. Classification of the cytokines and chemokines measured in this thesis.

Cytokine	Pro-inflammatory	Anti-inflammatory	Th1	Th2	Regulatory
IL-1β	x				
IL-6	x			x	
IL-10		x			x
IL-12	x		x		
TNF-α	x				
IFN-β	x				
IFN-γ			x		
Chemokine					
CCL19	x				
CCL20	x				
CXCL8	x				
CXCL10	x				

2.3.6 Regulation of cytokine production by SOCS3

Suppressor of cytokine signalling (SOCS) proteins are intracellular molecules induced in response to multiple stimuli including cytokines, growth factors, and TLR ligands. They interact with cytokine receptors and signalling molecules to regulate macrophage and DC activation and T cell differentiation and development via a negative feedback loop [131, 132]. SOCS proteins have been shown to regulate the responses of immune cells to cytokines. The expression of SOCS3 is regulated

by both anti-inflammatory and proinflammatory cytokines, especially IL-1β, TGF-β, IL-10, and IL-6 [133, 134]. SOCS proteins inhibit signalling through JAK/STAT pathway and participate in regulating the strength, duration, and specificity of cytokine responses (Figure 5). There are eight SOCS proteins in the human genome, all containing an N-terminal domain, a central SH2 domain which mediates SOCS binding to tyrosine phosphorylated signalling molecules, and a C-terminal SOCS box involved in targeting bound proteins to ubiquitination and proteasome degradation. SOCS1 and SOCS3 have shorter N-terminal domains than other family members. This allows them to interact directly with JAKs and inhibit their catalytic activity [131, 132].

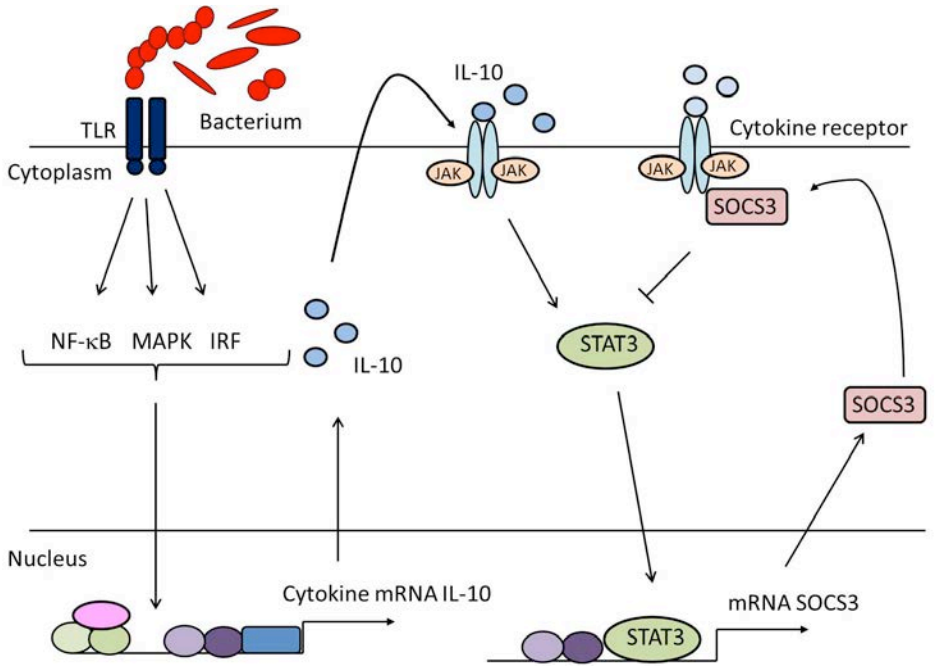

Figure 5. **Schematic representation of the feedback inhibition of cytokine signalling by SOCS3.** Ligand binding to TLRs result in the activation of transcription factors including NF-κB, MAPKs, and IRFs. After translocation into the nucleus these factors bind to the promoter element of cytokine genes, such as IL-10 and initiate mRNA transcription. IL-10 is produced and secreted and affects in an autocrine or paracrine fashion by binding to its receptor on the cell surface. Binding of IL-10 activates the JAK/STAT signalling pathway to activate SOCS3 gene expression. This ultimately leads to the binding of SOCS3 to the cytokine receptor chains which interferes with JAK activation, thus inhibiting JAK/STAT signalling.

2.4 Overview of probiotic bacteria

Probiotic bacteria are defined by the World Health Organization as 'live microorganisms which, when administered in adequate amounts, confer health benefit on the host' [135].

The probiotic strains which are used for human consumption are non-pathogenic bacteria mostly derived from beneficial commensals of human origin. For historical reasons most probiotics belong to the genera *Lactobacillus* and *Bifidobacterium*, which are generally used in dairy fermentation. Probiotic bacteria are thought to confer most of their health promoting activities in the gastrointestinal tract, where they can act through diverse mechanisms. They can improve barrier function, balance the composition or function of the commensal microbiota, inhibit the growth of other bacteria by secreting antimicrobial compounds, and compete for mucosal binding sites and nutrients with other bacteria. Probiotics can also mediate their beneficial effects via modulating the pro- and anti-inflammatory cytokine secretion by immune cells [136-139].

The possible beneficial health effects of probiotics on various diseases have been assessed in multiple clinical studies [140-142] and promising results have been obtained. *Lactobacillus rhamnosus* GG is one of the most intensively studied probiotic bacterium and several clinical studies support its beneficial effects on the host [143-149]. The effects of probiotic bacteria on the reduction of the risk of atopic diseases such as allergy and eczema have been extensively studied [136, 144, 145, 150] and potential beneficial effects have been observed. In addition, their ability to prevent or ameliorate the symptoms of various gastrointestinal disorders including rotaviral [143, 146, 148, 151] and antibiotic-associated [147] diarrhea have been studied. There are also promising results in amelioration of the symptoms of other gastroenterological disorders such as IBD and IBS [152-154]. The effects of probiotic bacteria in association with other than gut-related diseases such as dental caries, pancreatitis, vaginal and urinary tract infections, cancer and airway infections have also been suggested [155]. Despite these numerous studies with encouraging results, the molecular mechanisms behind these actions are less well understood.

Probiotic bacteria are usually non-pathogenic members of the normal gastrointestinal microbiota. In spite of a few reports of unfavourable effects in immunocompromised subjects, such as *Lactobacillus* septicaemia [156, 157] and detrimental effects on pancreatitis patients [158], probiotic therapy is generally considered as safe [159]. The use of bacterial strains in food is carefully evaluated and many *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Bifidobacterium, Streptococcus,*

and *Propionibacterium* strains have been determined safe by the European Food Safety Authority (EFSA) [160]. Although, probiotics are intensively studied, their safety as therapeutic substances has not been systemically examined. For example, many *Lactobacillus* strains carry antibiotic resistance genes [161] and there is a theoretical possibility that probiotic bacteria could transfer these genes to the commensal microbiota [162] or generate unfavourable metabolites in the intestine.

There are many challenges in probiotic clinical studies. The beneficial effects of probiotic bacteria are mostly characterized in immunocompromised or health-challenged persons. However, the baseline health status of a person likely affects the outcome in clinical trials. Less attention has been paid to their effects on healthy humans, who are most likely to consume probiotic products. In addition, healthy persons are shown to differ from allergic and IBS patients in their gut microbiota composition [163-167], which might affect the results. Moreover, the actions of probiotic bacteria are shown to be host-, strain-, and dose-specific and their biological effects cannot be concluded based on the results from another strain or species [168-170].

2.5 Overview of *Streptococcus pyogenes* pathogenicity

Streptococcus pyogenes (Group A streptococcus, GAS) is a Gram-positive human pathogen. It causes mostly diseases limited to the pharyngeal mucosa or skin such as tonsillitis and impetigo, but can also cause invasive infections such as erysipelas, sepsis, toxic shock syndrome, and necrotizing fasciitis. Although, mild non-invasive GAS infections can be easily cured by penicillin, GAS is among the ten most common causes of death from infectious disease worldwide. It is an important cause of morbidity and mortality worldwide with over 650 000 invasive GAS infections and 160 000 deaths each year [171]. Untreated infections can lead to severe post-streptococcal diseases such as glomerulonephritis and rheumatic fever [171, 172].

All GAS infections are characterized by a robust inflammatory reaction. The bacterium encodes multiple virulence factors that in various ways interfere with the host immune system and clearance mechanisms. These virulence factors can disrupt neutrophil recruitment to the infection site by degrading chemokines regulating neutrophil migration. In addition, virulence factors help the bacterium to avoid and escape the host surveillance system by e.g. preventing complement activation and interfering with the recognition or phagocytosis of the bacterium [173, 174]. Two important streptococcal virulence factors are the extracellular enzymes called streptolysins that can form pores in host cell membranes leading to apoptosis. In

addition, streptolysins facilitate the escape of the bacterium from the endosome-lysosome pathway [175, 176].

The streptococcal M protein is used for the serotypic classification of GAS. It is encoded by the *emm* gene of which more than 200 sequence types have been identified. The T1M1 serotype is one of the most frequently isolated serotypes in severe invasive GAS infections [173] and has the ability to protect the bacterium from phagocytosis by inhibiting complement deposition on the bacterial surface.

2.6 Sendai virus

Sendai virus, also known as murine parainfluenza type I, is an enveloped negative-stranded ssRNA virus causing severe respiratory infections in mice. It belongs to the genus *Respirovirus* of the *Paramyxoviridae* family. Sendai virus is considered non-pathogenic in humans [177, 178].

Sendai virus fuses directly with the plasma membrane to enter the host cells [179]. It replicates in the cytoplasm where virus-derived molecules such as viral nucleic acids can be detected by intracellular PRRs to activate intracellular signalling cascades. The strong antiviral responses induced by Sendai virus make it a useful tool for analysing host cell signalling pathways [178, 180, 181].

2.7 Interactions of bacteria with phagocytes in the intestine

One place where phagocytic DCs and macrophages come into contact with probiotic, commensal, and pathogenic bacteria is at the mucosal surfaces of the gut. The intestine is loaded with microbes and microbe-derived components and it is essential to limit the inflammatory responses induced in these areas of the body. In addition to capturing microbes that have crossed the epithelial barrier to the lamina propria, DCs can extend their appendices trough the tight junctions between epithelial cells and sample bacteria from the gut lumen via their cell surface receptors such as TLR2 and TLR4 [24, 182]. Probiotic bacteria are believed to have the ability to modulate the immune system in the gut. These effects may be elicited through direct interactions with the epithelial cells or resident DC and macrophage populations. The different abilities of probiotic bacteria to affect immune responses may be associated with bacterial cell wall structure, secreted bacterial factors, or their unmethylated CpG DNA content [183-187].

The intestinal immune system is the largest and most complex part of the immune system protecting against potential pathogens and giving tolerance to ingested food antigens and microbiota that inhabits the gut [5]. The challenge is to discriminate harmless antigens such as commensal bacteria and food antigens from invasive microorganisms and these mechanisms remain to be elucidated. However, the differential distribution of TLRs on the apical and basolateral surfaces of epithelial cells helps to detect bacteria that have crossed the epithelial cell layer and to initiate appropriatae immune responses [188, 189]. In addition, the suppressed NF-κB activation from apical surfaces might play a role in gut homeaostasis [189].

3 Aims of the study

Macrophages and DCs play a central role in the immune responses induced by microbes. They are the key cell types under the epithelial cell layers throughout the body and sample the environment in case of intruders. Despite intensive research in recent years, the mechanisms how probiotic bacteria elicit their health promoting effects are poorly characterized. In addition, the mechanisms of *Streptococcus pyogenes* infections are not fully understood.

The specific objectives of this study were:

1. To analyse and compare the cytokine responses induced by multiple probiotic bacteria, potentially probiotic bacteria, and pathogenic *S. pyogenes* in DCs and macrophages.

2. To study the role of anti-inflammatory IL-10 in the responses induced by non-pathogenic bacteria.

3. To study the mechanisms of *S. pyogenes* -induced inflammatory responses and the role of streptococcal virulence factors and dynamin-dependent endocytosis in the activation of host innate immune responses.

4 Materials and methods

4.1 Human primary cell cultures

4.1.1 Monocyte-derived macrophages and dendritic cells (DCs)

Leukocyte-rich buffy coats from healthy blood donors were supplied by the Finnish Red Cross Blood Transfusion Service (Helsinki, Finland). Each experiment was carried out with cells obtained from three or four blood donors. Human peripheral blood mononuclear cells (PBMC) were isolated by density-gradient centrifugation over a Ficoll-Pague Gradient (Amersham Biotech, Uppsala, Sweden) and washed with phosphate buffered saline (PBS). Monocytes were purified from PBMCs by allowing them to adhere to plastic plates for 1 h at 37°C, after which non-adherent cells were removed by washing with PBS.

To differentiate monocytes into macrophages, cells were cultivated for 7 days in serum-free macrophage medium (Gibco) supplemented with antibiotics (penicillin 0.6 μg/ml, streptomysin 60 μg/ml) and GM-CSF (10 ng/ml) (Biosource, Camarillo, CA, USA). Fresh medium was changed every two days.

To obtain moDCs, PBMCs were further purified by Percoll-gradient centrifugation and magnetic depletion (Dynal, Oslo, Norway) of contaminating T and B cells. Cells were then allowed to adhere to plastic plates for 1 h at 37°C, after which non-adherent cells were removed by washing with PBS. Cells were cultivated for 6-7 days in RPMI-1640 medium supplemented with antibiotics (penicillin 0.6 μg/ml, streptomysin 60 μg/ml), 2mM glutamine, 20 mM HEPES, 10 % FCS, 10 ng/ml GM-CSF, and 20 ng/ml IL-4. Fresh medium was added every two days (Figure 6).

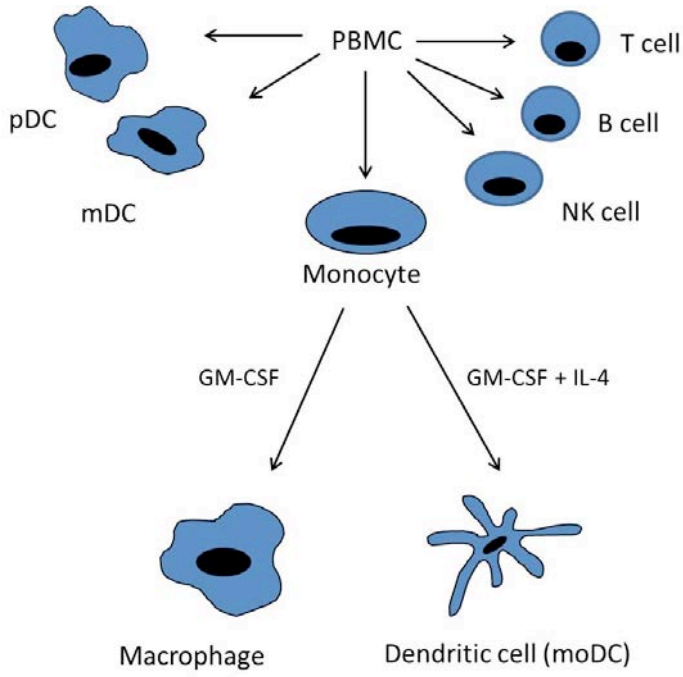

Figure 6. Differentiation of macrophages and dendritic cells from monocytes.

4.2 Microbes

4.2.1 Probiotic and potentially probiotic bacteria

Five probiotic strains and six potentially probiotic bacterial strains were obtained from Valio Research Centre (Helsinki, Finland). Bacteria were stored in skimmed milk at -70°C and passaged three times (except for *Bifidobacterium* strains which were passaged four times) before they were used in experiments. Bacteria were grown to the end of logarithmic growth phase before they were collected by centrifugation and used in experiments. The number of bacteria was determined by counting in a Petroff-Hausser chamber. Bacterial strains used in this thesis are described in tables 3 and 4.

Table 3. Non-pathogenic bacteria used in these studies.

Bacterial species/ subspecies	Abbreviation	ATCC/DSM number	Culture media	Growth conditions	Use
Bifidobacterium					
Bifidobacterium animalis subsp. *lactis* Bb12	Bb12	DSM 15954	MRS medium[1] with cysteine[2]	+ 37°C, anaerobic	probiotic
Bifidobacterium breve Bb99	Bb99	DSM 13692			probiotic
Bifidobacterium longum 1/10	1/10	NA			none
Lactobacillus					
Lactobacillus rhamnosus GG	LGG	ATCC 53103	MRS medium[1]	+ 37°C, aerobic	probiotic
Lactobacillus rhamnosus LC705	LC705	DSM 7061		+ 37°C, aerobic	probiotic
Lactobacillus helveticus 1129	1129	DSM 13137		+ 42°C, aerobic	cheese, fermented milk
Lactobacillus helveticus Lb 161	161	NA		+ 42°C, aerobic	cheese
Lactococcus					
Lactococcus lactis subsp. *cremoris* ARH74	ARH74	DSM 18891	Calciumcitrate agar[3] and M17 broth[4] with lactose[5]	+ 37°C, aerobic	sour whole milk product (viili)
Leuconostoc					
Leuconostoc mesenteroides subsp. *cremoris* PIA2	PIA2	DSM 18892	MRS medium[1]	+ 22°C, aerobic	sour whole milk product (viili)
Propionibacterium					
Propionibacterium freudenreichii subsp. *shermanii* JS	PJS	DSM 7067	Propionimedium[3]	+ 30°C, aerobic	probiotic, cheese
Streptococcus					
Streptococcus thermophilus THS	THS	NA	M17 agar[1] with lactose[5] and M17 broth[4]	+ 37°C, aerobic	yoghurt

ATCC, American Type Culture Collection; **DSM**, German Collection of Microorganisms; **MRS**, de Man, Rogosa and Sharpe medium.[1]Lab M, Topley House, Lancashire UK; [2]Merck, Darmstadt, Germany; [3]Valio Ltd, Helsinki, Finland; [4]Difco, Becton Dickinson, MD, USA; [5]J.T. Baker B.V., Deventer, Holland.

4.2.2 *Streptococcus pyogenes*

Pathogenic *Streptococcus pyogenes* serotype T1M1 (IH32030) [190], isolated from a child with bacteremia, was from the collection of the National Institute for Health and Welfare (Helsinki, Finland). *Streptococcus pyogenes* M1 (ATCC 700294) is a clinical strain originally isolated from infected wound. *S. pyogenes* mutants lacking *slo* (EC997) [191] or *sagA* (EC695), the genes encoding major streptococcal virulence factors streptolysin O (SLO) and streptolysin S (SLS), respectively, were provided by Emmanuelle Charpentier (Umeå University, Sweden). Heat inactivation of bacteria was performed in 95°C dry heat block for 5 min and UV inactivation by exposing bacteria to UV light at an energy level of 2 x 0,12J. Complete inactivation of bacteria was verified by sheep blood agar plate counting method. *S. pyogenes* – strains used in the study are summarized in table 4.

Table 4. Pathogenic bacteria used in these studies.

Bacterial strains	Abbreviation	ATCC number/source	Culture media	Growth conditions	Used in study
Streptococcus pyogenes (IH32030)	GAS	National Institute for Health and Welfare	Sheep blood agar[1], Nefrit broth[2]	+ 37°C aerobic	I, II
Streptococcus pyogenes	GAS	ATCC 700294			III
Streptococcus pyogenes (EC997)	SLO$^-$	E. Charpentier			III
Streptococcus pyogenes (EC695)	SLS$^-$	E. Charpentier			III

[1]Oxoid, Ogdensburg, NY, USA; [2]National Institute for Health and Welfare, Helsinki, Finland.

4.2.3 Sendai virus

Sendai virus (Cantell strain, National Institute for Health and Welfare, Helsinki, Finland) was grown in embryonated chicken eggs and stored in -70°C. The hemagglutination titer of the virus was 4096 and the infectivity of the stock in macrophages and DCs was 6 x 10^9 pfu/ml. Cells were infected with multiplicity of infection (MOI) value of 5.

4.3 Reagents

4.3.1 Cytokines

For priming experiments, cytokines were added to cell culture media 16 h prior to bacterial stimulation. Cytokines used in the thesis are summarized in table 5.

Table 5. Cytokines used in these studies.

Cytokine	Concentration used	Source	Used in publication
TNF-α	10 ng/ml	Biosource	I
IL-6	10 ng/ml	Biosource	I
IL-10	10 ng/ml	R&D Systems	II
IFN-γ	100 IU/ml	Finnish Red Cross	I

4.3.2 Inhibitors

Commercial pharmacological inhibitors were used to inhibit cell signalling pathways and other cellular processes. Inhibitors and other reagents used in the experiments are summarized in table 6.

Table 6. Pharmacological inhibitors and other reagents used in these studies.

Signalling inhibitor	Concentration used	Target	Source	Used in publication
CsA	0.2 or 1 μg/ml	NFAT	Alexis Biochemicals	I
Ly294002	10 or 50 μM	PI3K	Calbiochem	I,II
SB202190	2 or 10 μM	p38 MAPK	Calbiochem	I, II
SP600125	10 μM	JNK MAPK	Alexis Biochemicals	II
PD98059	2 or 10 μM	ERK MAPK	Calbiochem	I, II
PDTC	50 or 100 μM	NF-κB	Alexis Biochemicals	I, II
Other inhibitors				
CHX	30 μg/ml	de novo protein synthesis	Sigma	II
Dynasore	80 μM	dynamin [85]	Sigma	III
Other reagents				
Pam_3CSK_4	100 ng/ml	TLR1/2 ligand	InvivoGen	II
LPS (0111:B4)	100 ng/ml	TLR4 ligand	Sigma	II
anti-IL10	1 μg/ml	IL-10	R&D Systems	II

CHX, cycloheximide; **CsA**, cyclosporine A; **LPS**, lipopolysaccharide; **MAPK**, mitogen activated protein kinase; **NFAT**, nuclear factor of activated T cells; **NF-κB**, nuclear factor kappa-B; **Pam_3CSK_4**, tripalmitoyl-S-glyceryl-cysteine; **PDTC**, pyrrolidine dithiocarbamate; **PI3K**, phosphoinositide 3-kinase.

4.4 mRNA analyses

4.4.1 Northern blot analysis

Total cellular RNA was isolated by using an RNA purification kit (Qiagen). Samples containing equal amounts of RNA were separated by size on 1% formaldehyde-agarose gels and transferred onto Hybond-N-membranes (Amersham Biosciences). Membranes were hybridized with human cytokine cDNA probes labelled with [α-^{32}P] deoxy-adenosine 5'-triphosphate (3000 Ci/mmol, Amersham Pharmacia Biotech) using a random-primed DNA labelling kit (Fermentas). Membranes were hybridized, washed, and exposed to Kodak X-Omat AR-films.

4.4.2 Quantitative RT-PCR

Total cellular RNA was isolated with an RNA purification kit (Qiagen). Synthesis of cDNA was performed using Multiscribe RT (Applied Biosystems) with oligo-d(T) primers. Amplification of cDNA was performed using Master Mix Buffer with Assays-on-Demand gene expression primers and probes (Applied Biosystems). Samples were amplified in duplicate with MxPro 3005P (Stratagene) and normalized against β-actin. Relative amounts of mRNA were calculated with the ΔΔ comparative threshold (Ct) method [192].

4.5 Protein analyses

4.5.1 ELISA

Cytokine levels from cell culture supernatants were analysed by a sandwich ELISA method with antibody pairs and standards obtained from BD Pharmingen, R&D Biosystems or Biosite. Also a FlowCytomix bead assay (Bender Medsystems) was used for cytokine measurements.

4.5.2 Flow cytometry

Cells were collected, washed with PBS, fixed with paraformaldehyde, and stored in 2% FCS/PBS at 4°C before staining. Cell surface protein markers were stained with FITC- and PE-conjugated monoclonal antibodies and isotype mached control antibodies (Caltag Laboratories). Samples were analyzed with FACScan cytometer and Cell Quest software (Becton Bickinson).

4.5.3 Western blot analysis

Whole cell lysates were prepared in Laemmli sample buffer for Western blot analysis. The proteins were separated on 15% SDS-PAGE, transferred onto Immobilon-P membranes (Millipore), and stained with specific antibodies.

5 Results and discussion

The results of this study highlight the complex interactions between the host, commensal microbiota, and pathogenic bacteria. We have approached this issue with *in vitro* experiments that enable to study a selected microbe in highly controlled experimental conditions and the analysis of their effects at molecular and cellular level. It must be, however, taken into account that *in vitro* differentiated cells and cell lines may differ from their *in vivo* counterparts when taken away from their original milieu including normal microbiota, epithelial cells, and other blood cells. In addition, a disadvantage of *in vitro* studies is their limited duration. Despite these limitations, the advantages of *in vitro* studies are undeniable. In this study, our goal was to clarify the mechanisms of the beneficial effects of probiotics on the host by using human *in vitro* differentiated macrophages and DCs that most likely encounter these microbes *in vivo*. In addition, the ability of GAS and its virulence factor defective mutant derivatives to initiate innate immune responses and the role of dynamin-dependent endocytosis in these events were also analysed. The cells used in this study resemble immature DCs and macrophages that are recruited from blood to the infection site. Mucosal DCs primarily consist of myeloid DCs, which are likely of monocyte origin.

5.1 Maturation of DCs (I)

Immature DCs mature into APCs upon encountering microbes or foreign antigens, in this case different species of non-pathogenic or pathogenic bacteria. The interactions of host cells with bacteria most likely take place at the mucosal surfaces of the gastrointestinal and respiratory tract or through an injury in the mucosal epithelial cell layer or the skin. Non-pathogenic or pathogenic bacteria can be ingested by macrophages and DCs to initiate innate and adaptive immune responses. Mature DCs secrete cytokines and chemokines and express cell surface molecules such as HLA class II and costimulatory molecules CD80, CD83, and CD86. In addition to their role in innate immunity, matured DCs migrate from peripheral tissues into local lymph nodes to present antigens to naïve T cells to initiate adaptive immune responses.

This study was initiated since previous studies had provided evidence on the ability of pathogenic bacteria and their purified components to enhance DC maturation [193-195]. There was also data available on the ability of non-pathogenic

lactobacilli and bifidobacteria [168, 196-199] including LGG [200] to modulate human DC functions. Because of the growing interest towards probiotic bacteria, we wanted to compare the ability of other probiotic and potentially probiotic Gram-positive bacteria used in food industry to stimulate human DCs. The ability of various bacteria to induce DC maturation, cytokine production, and the expression of other inflammatory genes was analysed and compared with those induced by pathogenic GAS.

We went on to study the ability of various non-pathogenic bacteria to induce human DC maturation. In a previous report it was shown that both the *Lactobacillus rhamnosus* strain LGG and pathogenic GAS were able to induce DC maturation [200]. Although, both LGG and GAS clearly induced CD83 and CD86 expression on the cell surface, LGG-stimulated cells showed lower expression levels compared with GAS-stimulated cells. It was of interest that LGG could efficiently induce DC maturation and therefore we analysed whether other non-pathogenic bacteria had the same ability. We showed that non-pathogenic bacteria representing *Streptococcus*, *Bifidobacterium,* and *Lactococcus* genera induced DC maturation as efficiently as pathogenic GAS as evidenced by increased expression of CD86 and HLA class II molecules on the cell surface (Fig. 3 in I). These molecules were induced in a bacterial dose-dependent manner already with a low bacteria:DC ratio of 2:1. However, it has been reported previously that different clinical GAS isolates have distinct abilities to induce DC maturation. GAS strains that produced large amounts of SLO and hyaluronic acid capsular polysaccharide failed to induce DC maturation [201]. Thus, our results and those by others suggest that the expression of DC maturation markers can be upregulated by both pathogenic and non-pathogenic bacteria and the ability of some bacterial strains to inhibit DC maturation could be a feature of some pathogenic bacteria or bacterial strains to avoid host immune responses. Our results showing the expression of costimulatory molecule CD86 in addition to HLA class II suggests that also DCs stimulated with non-pathogenic bacteria would be able to present antigens to T cells and induce their polarization [202]. Our data is supported by another study where inactivated *Lactobacillus* species [169] were shown to modulate the expression of surface maturation markers and cytokine production in murine DCs. In addition, another report showed that DC maturation markers, especially CD86 and HLA class II were upregulated in human DCs in response to live and irradiated bacteria belonging to *Lactobacillus*, *Bifidobacterium*, and *Streptococcus* species [203].

5.2 Cytokine responses in human primary cells

Both DCs and macrophages produce cytokines and chemokines in response to microbial stimulation. However, the amount and repertoire of cytokines differ depending on the cell type and bacterial strain and dose used to stimulate the cells.

5.2.1 Comparison of DC and macrophage cytokine responses (I, II)

The mammalian immune system has the ability to launch distinct types of immune responses against different microbes. In addition to the upregulation of costimulatory molecules, the maturation process of DCs includes the secretion of cytokines and chemokines in response to antigen stimulation or microbial infection. When this study was commenced, there were no reports on the effects of the studied bacteria, other than LGG, on human DCs. Previously, the cytokine profiles generated by exposure to these bacteria [204] and to another set of multiple non-pathogenic bacteria [205] were analysed in human PBMCs. To our knowledge, we were the first ones to screen the cytokine profiles and inflammatory responses induced by multiple probiotic or potentially probiotic bacteria in the human DC model system.

In addition to maturation markers (Fig. 3 in I), some non-pathogenic bacteria were able to stimulate cytokine and chemokine production in DCs (Fig. 1-2 in I). Previously, the ability of LGG to stimulate cytokine and chemokine production in human DCs [200] and macrophages [113, 206] had been analysed, but other non-pathogenic bacteria were not included in these studies. In our studies DCs showed more versatile cytokine profiles to different bacteria than macrophages (Fig. 1 in I and Fig. 5). In agreement with the previous study by Veckman and coworkers [200], LGG was quite inert in inducing cytokines in human DCs. This was also the case with another *Lactobacillus rhamnosus* strain LC705, while for example *Bifidobacterium* strains Bb12 and Bb99 efficiently induced production of all the measured cytokines (TNF-α, IL-1β, IL-6, IL-10, IL-12, and IFN-γ). *Streptococcus thermophilus* THS on the other hand induced proinflammatory cytokines TNF-α, IL-6, and IL-12 but no IL-1β or anti-inflammatory IL-10. We concluded that although DC maturation marker expression is similarly induced in response to bacteria representing different genera, the cytokine production profiles can vary greatly between the bacteria. It might be that these bacteria induce different stages of DC maturation. DCs expressing moderate levels of costimulatory molecules but having restricted ability to induce cytokine responses are considered as semi-mature DCs [19, 200] that may be involved in the induction of regulatory T cells [207]. Thus, in our DC model system at least LGG and LC705 could support the

generation of regulatory T cells as suggested by their increased maturation marker expression [200] and low capacity to stimulate cytokine production (Fig. 1 in I).

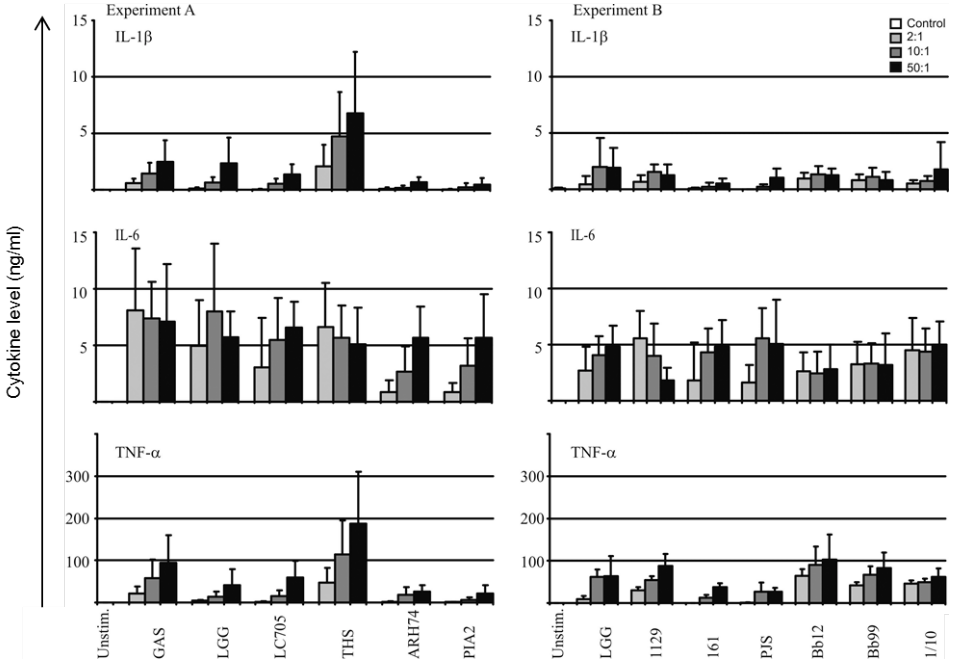

Figure 7. **Production of proinflammatory cytokines IL-1β, IL-6, and TNF-α in macrophages stimulated with different bacteria.** Macrophages were stimulated with different doses of bacteria (bacteria:macrophage ratios of 2:1, 10:1, and 50:1) for 24 h and the cytokine levels were analysed from cell culture supernatants by ELISA. Macrophages seem to respond strongly to bacterial stimulation and produce large amounts of proinflammatory cytokines. **GAS**, *Streptococcus pyogenes*; **LGG**, *Lactobacillus rhamnosus* GG; **LC705**, *Lactobacillus rhamnosus* LC705; **THS**, *Streptococcus thermophilus* THS; **ARH74**, *Lactococcus lactis* subsp. *cremoris*; **PIA2**, *Leuconostoc mesenteroides* subsp. *cremoris* PIA2; **1129**, *Lactobacillus helveticus* 1129; **161**, *Lactobacillus helveticus* Lb161; **PJS**, *Propionibacterium freudenreichii* subsp. *shermanii* JS; **Bb12**, *Bifidobacterium animalis* subsp. *lactis* Bb12; **Bb99**, *Bifidobacterium breve* Bb99; **1/10**, *Bifidobacterium longum* 1/10.

Bifidobacterium strains Bb12 and Bb99 induced production of all the measured cytokines, including anti-inflammatory IL-10 in DCs (Fig. 1 in I). The ability of bifidobacteria or their secreted compounds to induce IL-10 has been characterized

before in *in vitro* cell models and clinical trials [168, 187, 205]. Since IL-10 has the ability to suppress proinflammatory cytokine production it might be one factor contributing to the observed immunomodulatory effects of bifidobacteria [208]. IL-10 is generally accepted to be involved in the generation of regulatory T cells [15]. In addition to IL-10 production in DCs, we observed the ability of bifidobacteria to induce IL-10 production in human macrophages (Fig. 1 in II), suggesting that both cell types participate in the regulation of inflammatory responses via IL-10 production.

The production of proinflammatory TNF-α and IL-6 was induced efficiently by all bacteria in macrophages and the levels were higher compared with the cytokine levels in bacteria-stimulated DCs (Fig. 1 in I and Fig. 7). Previously the ability of LGG to induce TNF-α and IL-6 in macrophages was analysed [113] and these results are in line with the ones presented now. However, LGG and LC705 were unable to induce TNF-α production in DCs. TNF-α is known to promote inflammatory responses by upregulating many inflammatory molecules including other cytokines [209]. Our results indicate that macrophages respond with a strong proinflammatory cytokine pattern to all bacteria, while DCs are more selective. Thus, these two cell types probably have distinct roles in the initiation of immune responses against microbes. This supports the role of macrophages in being an important cell type regulating inflammatory responses in tissues. The importance of macrophages in the early control of infections has been previously characterized [210].

The production of IL-1β is used as an indicator of inflammasome activation. In contrast to macrophages that induced high levels of IL-1β in response to bacterial stimulation (Fig. 7), in DCs only four non-pathogenic bacterial strains were able to induced inflammasome activation (Fig. 1 in I). IL-1β was efficiently induced in DCs after stimulation with *Bifidobacterium* strains Bb12 and Bb99 as well as with *Lactococcus lactis* ARH74 and *Lactobacillus helveticus* 1129 while other strains were unable to stimulate IL-1β production. It is interesting that the pathogenic GAS could not induce IL-1β production and inflammasome activation in DCs but induced it efficiently in macrophages. The data suggests that the inflammasome complex is activated in macrophages in response to all kinds of bacterial stimulation while only a few bacteria are able to activate the inflammasome complex in DCs, thus caspase-1 activation and cleavage of pro-IL-1β into its active secreted form.

Our data shows that DCs produce differential patterns of cytokines in response to various bacterial strains. Macrophages on the other hand respond more strongly to bacterial stimulation with less dramatic differences between bacteria. (Fig. 1 in I, Fig. 1 in II, and Fig. 7). Thus, the immunomodulatory effects of probiotic bacteria

may be due to their ability to stimulate the production of cytokines in immune cells such as macrophages and DCs. These stimulatory properties are likely to differ between bacteria since different bacterial strains possess diverse MAMPs, secreted bacterial proteins, or other metabolites as previously shown [183, 185-187, 211]. Most of the studies show the favorable effects of bacterial soluble proteins on intestinal epithelial cell growth and survival. However, in human macrophages [212] and murine DCs [169] also inactivated *Lactobacillus* strains were able to stimulate cytokine production. These data suggest that no active metabolism or secreted bacterial components are required for human macrophage or murine DC cytokine responses to these *Lactobacillus* strains. Thus, the inflammatory responses and cytokine profiles induced by different bacteria are likely due to interactions of bacterial surface components with their cognate receptors and only in some cases secreted bacterial metabolites are needed to mediate the inflammatory responses.

5.2.2 Comparison of the cytokine production profiles of non-pathogenic and pathogenic bacteria (I, II)

Compared with pathogenic GAS, some non-pathogenic bacteria induced cytokine production more efficiently in DCs (Fig. 1 in I). For example proinflammatory IL-1β was practically not detected from GAS-stimulated DCs, while some of the non-pathogenic bacterial strains belonging to *Bifidobacterium*, *Lactobacillus,* or *Lactococcus* genera efficiently induced IL-1β production. The inability of this GAS strain to induce IL-1β secretion might be a mechanism of pathogenesis and a concequence of the actions of bacterial virulence factors. However, GAS-stimulation induced DC maturation and the production of proinflammatory cytokines such as TNF-α, IL-6, IL-12, and IFN-γ. This suggests that although the inflammasome complex is not activated in DCs after GAS stimulation, the bacterium is able to trigger other proinflammatory responses. It is interesting that there were also non-pathogenic bacteria such as the two *Lactobacillus* strains LGG and LC705, *Streptococcus thermophilus* THS, *Leuconostoc mesenteroides* PIA2, and *Bifidobacterium longum* 1/10, which were unable to induce IL-1β production. The non-pathogenic *Streptococcus thermophilus* THS induced mainly proinflammatory cytokines TNF-α, IL-6, and IL-12 in DCs, showing a similar trend with GAS in its ability to induce cytokine production in DCs. However, the cytokine levels were higher than in GAS-stimulated DCs. Thus, both the pathogenic GAS and some non-pathogenic bacteria belonging to different genera were unable to activate the inflammasome in DCs. This suggests that the inability to activate the inflammasome is not always related to the pathogenicity of the bacterium. The bacterial factors and mechanisms behind these phenomena remain unknown and they likely differ between bacterial strains. This would be an interesting topic for further research.

The production of IL-10 in DCs after bacterial stimulation had a similar trend as IL-1β production. Thus, the same bacteria were able or unable to iduce IL-1β and IL-10 production (Fig 1 in I). IL-10 suppresses the production of proinflammatory cytokines especially IL-12 [101, 102]. The strong proinflammatory responses in GAS infections could be related to the lack of IL-10 production and its suppressive effects on proinflammatory responses. The non-pathogenic bacteria which were unable to induce IL-10 production (*Lactobacillus* strains LGG and LC705, *Streptococcus thermophilus* THS, *Leuconostoc mesenteroides* PIA2 and one *Bifidobacterium* strain 1/10) were also poor inducers of proinflammatory TNF-α, IL-1β, IL-12, and IFN-γ, except for *Streptococcus thermophilus* THS which strongly induced TNF-α, IL-6, and IL-12 production. It is interesting that the non-pathogenic *Streptococcus* strain induced more efficient inflammatory responses than pathogenic GAS in DCs.

In macrophages the differences in proinflammatory cytokine levels between the studied bacteria were not that clear, and all bacteria were able to induce production of the measured proinflammatory cytokines IL-1β, IL-6 and TNF-α (Fig. 7). However, some differences were seen in IL-12 and IL-10 production levels (Fig 1 in II). This data highlights the strain-specific effects of different bacteria which have also been characterized in other studies [169, 203, 205, 213]. Thus, based on our studies and those by others, the immune responses induced by bacteria must be characterized individually for each bacterial strain.

5.2.3 The anti-inflammatory potential of bacteria - IL-10/IL-12 ratio (II)

It is widely accepted that the gut microbiota has an immunomodulatory capacity and play an important role in the health of the host. It has been suggested that the balance between Th1 and Th2 immunity as well as immune tolerance may be controlled by DCs [214, 215]. The capacity of bacterial strains to induce different cytokine profiles suggests that bacteria may differentially determine DC polarization patterns. Thus, bacteria can be classified as anti-inflammatory Th2 -type or inflammatory Th1 -type strains based on their ability to stimulate cytokine production. IL-12 is an important cytokine in inducing Th1 responses by eliciting IFN-γ production by T cells and NK cells [216]. Anti-inflammatory IL-10 favors Th2 or Treg responses via suppression of IL-12 production [217]. Since, IL-10 has the ability to downregulate the production of IL-12 [205, 218, 219], the IL-10/IL-12 ratio can be used as an indicator for the immunomodulatory potential of a bacterium [168, 220]. There are reports showing that probiotic bacteria are able to modulate the Th1/Th2 balance towards Th1, which may downregulate overactive Th2-

mediated allergic responses [221, 222]. On the contrary in Th1-mediated autoimmune conditions such as IBS, probiotic treatment can balance the Th1/Th2 ratio towards Th2 responses [208].

In DCs and macrophages some bacteria can induce the production of anti-inflammatory IL-10 while others induce modest or undetectable levels of IL-10 (Fig. 1 in I, Fig. 1 in II). Also great differences in IL-12 production between the studied bacteria were seen. The most potent IL-10 inducers were not the same bacterial species in both cell types. For example LGG induced IL-10 production in macrophages but not in DCs, THS in turn induced both IL-10 and IL-12 in macrophages but only IL-12 in DCs. Although, the effects of probiotic bacteria seem to be strain specific, previous studies show that many *Bifidobacterium* strains are potent inducers of IL-10 [168, 204, 223]. In our studies bifidobacteria were efficient inducers of IL-10 in macrophages and DCs, which might explain their anti-inflammatory effects in clinical trials [208].

Macrophages are often divided into classically or alternatively activated macrophages based on their IL-10/IL-12 production ratio [224, 225]. Classically activated macrophages are an important source of IL-12 [219], while alternatively activated macrophages produce higher levels of IL-10 and reduced levels of IL-12. As shown in our studies with macrophages, bifidobacteria have high anti-inflammatory potential (Fig. 2 in II). Many clinical studies also suggest that LGG has immunomodulatory potential [144, 145, 149, 150, 204, 226]. This is supported by our results showing that *Lactobacillus rhamnosus* strains LGG and LC705 have relatively high anti-inflammatory potential in human macrophages.

Excessive production of Th1 cytokines can contribute to tissue destruction and development of autoimmune diseases such as multiple sclerosis, diabetes, and systemic lupus erythematosus (SLE). However, Th1 cytokines may be protective against Th2-related chronic inflammatory conditions including asthma and atopy. It has been proposed that probiotic-induced low-grade inflammation could protect from atopic eczema [222]. Gastrointestinal disorders such as Crohn's disease are linked to the predominance of Th1 cytokines while ulcerative colitis is associated with production of Th2 cytokines such as IL-5. It would be desirable to modulate the unwanted immune responses in these conditions with administration of probiotic strains which are able to modulate the Th1/Th2 cytokine balance of the host. Based on our results, the two *Bifidobacterium* strains Bb12 and Bb99 and *L. helveticus* 1129 are probably the most potent inducers of anti-inflammatory responses, based on their ability to induce IL-10 production in both cell types and their IL-10/IL-12 ratio in macrophages (Figure I in I, Figs. 1 and 2 in II). The *Bifidobacterium* strain Bb12 is widely used in dairy products in Europe, extensively studied, and previously

shown to limit the duration of diarrhea [227-229]. Bb99 has been used in a probiotic mixture which was able to alleviate the symptoms of IBS [152, 153]. *L. helveticus* 1129 has been previously studied only in vitro [204, 230] and no clinical evidence of its immunomodulatory effects is available. Thus, it would be an interesting bacterium for further research.

The immunomodulatory properties of a particular bacterium can vary with different bacterial doses [223]. IL-10 is induced more efficiently with a high bacterial dose [169] suggesting that the optimal bacterial dose for each cytokine must be individually determined. The anti-inflammatory potential of a bacterium is most likely a result of complicated interactions with epithelial cells, immune cells, and commensal microbes combined with the cytokine milieu in different tissues. These *in vitro* results of the present studies give some guidance how to select the potential anti-inflammatory bacterial strains, and point out possible new bacterial strains that have not yet a probiotic status for clinical trials.

5.2.4 Probiotic-induced SOCS3 expression and regulation of cytokine responses (II)

SOCS proteins, the negative regulators of cytokine signalling, are inducible upon cytokine stimulation and implement their regulatory properties by interfering with the JAK/STAT pathway [131]. The expression of SOCS3 is known to be regulated by both anti-inflammatory IL-10 and proinflammatory cytokines to control cytokine expression [134, 231, 232].

In our studies with DCs, non-pathogenic bacteria, especially the *Bifidobacterium* strains, were able to induce IL-10 production more efficiently than other bacteria. This directed our interest to the regulatory mechanism behind the proposed anti-inflammatory effects of bifidobacteria and the role of IL-10 production in these events. At the time we started to study the role of SOCS3 in the immune responses induced by probiotic and other non-pathogenic bacteria, to our knowledge, no publications were available on the role of SOCS3 in human immune cell responses to probiotic stimulation. During our studies, the role of SOCS3 in bifidobacteria-stimulated mouse macrophage cell line [233], and later on a report about the probiotic actions mediated by SOCS3 during *Helicobacter pylori* infection in a human gastric carcinoma cell line were published [234].

Non-pathogenic bacteria were able to induce SOCS3 gene expression in macrophages (Fig. 3 in II). As known from previous studies [231, 235], SOCS3 is upregulated by IL-10, and as expected, the ability of a bacterium to induce IL-10

correlated with SOCS3 expression levels in our macrophage model as well. The SOCS3 gene expression was induced by bacteria at 8 h after stimulation in the absence of protein synthesis (Fig. 4 in II). Thus, SOCS3 mRNA expression is a direct consequence of bacteria-macrophage interaction. SOCS3 mRNA expression increased up to 24 h after stimulation suggesting that a positive feedback loop might exist (Fig. 3 in II). Since neutralising IL-10 antibodies were able to decrease bacteria-induced SOCS3 mRNA expression we concluded that SOCS3 expression is enhanced also indirectly via bacteria-induced IL-10 production (Fig. 6 in II). The immunoregulatory effects of LGG seen in clinical studies as decreased production of IL-6 and TNF-α in healthy individuals [226] and reduction in TNF-α production in allergic children [150] may partly be regulated by SOCS3. Our data shows that LGG-stimulation upregulates SOCS3 mRNA expression (Fig. 3 in II). Since LGG activates STAT1 and STAT3 DNA binding [113] and STAT3 is known to be involved in IL-10 induced SOCS3 expression in human macrophages [236] it is likely that SOCS3 is a negative regulator of probiotic-induced cytokine responses via IL-10 production and inhibition of the JAK/STAT pathway (Figure 5).

Genes of many proinflammatory cytokines such as IL-1β, TNF-α, and IL-12 are regulated by NF-κB. It was previously shown that bifidobacteria decreased LPS-induced inhibitor of NF-κB (IκB)-α phosphorylation in a macrophage cell line [233], which is probably one factor contributing to their anti-inflammatory effects. Additionally, lactobacilli were shown to have similar effects [234, 237]. Thus, the anti-inflammatory effects seen in clinical trials, animal studies, and cell cultures in response to probiotic bacteria are most likely due to a combination of different regulatory mechanisms. These involve the suppression of proinflammatory cytokine production by inhibiting NF-κB activation and induction of inhibitory molecules such as SOCS to further downregulate the signalling cascades involved in these inflammatory responses. Modifying the SOCS3 pathway could offer a new therapeutic approach to the management of some autoimmune diseases such as IBD.

5.2.5 Contribution of streptococcal viability and bacterial virulence factors to innate immune responses (III)

There are conflicting reports on the role of bacterial viability in the immune responses they induce *in vitro*. The impaired ability of heat-inactivated GAS to induce cytokine production in human DCs was previously characterized [200]. However, also opposing data in support of similar immunostimulatory potential of live and dead bacteria exist on human macrophages [212]. Our data from macrophages stimulated with live and inactivated GAS (Fig 1 in III) showing similar cytokine responses at later time point of infection, suggests that GAS does not

secrete metabolites that induce or inhibit inflammatory responses in macrophages and DCs. Heat-inactivated bacteria induced lower cytokine mRNA levels in macrophages than live GAS at early time points of stimulation, but the cytokine levels were comparable to live bacteria at later time points (Figs. 1, 2, and 3 in III). We conclude that heat-inactivation of bacteria might modulate bacterial surface molecules and result in differential recognition by immune cells compared with viable bacteria, thus leading to differences in the kinetics of cytokine mRNA expression. This data shows that bacterial viability does not contribute to final outcome of the immune responses induced in human macrophages by GAS stimulation (Fig. 1 in III).

Streptococcal virulence factors, streptolysins, have a role in GAS pathogenicity and increased streptolysin O (SLO) expression has been detected in GAS strains isolated from invasive infections [173]. SLO is a cholesterol-dependent cytolysin that participates in the formation of transport vesicles and their fusion with endosomes. It is shown to block clathrin-dependent pathway for internalization of GAS in human keratinocytes and it is involved in the inhibition of lysosomal degradation of GAS. Thus, helping the bacterium to escape into the cytoplasm to avoid lysosomal killing [238, 239]. Instead, a mutant bacterium lacking functional SLO was internalized into lysosomes in human keratinocytes [239]. SLO can form pores in host cell membranes and the gene encoding SLO is co-transcribed with another bacterial factor, nicotinamide adenine dinucleotide (NAD^+)-glycohydrolase (SPN), which is translocated with SLO into the cytoplasm of target cells to deplete the host cell energy stores [240]. Streptolysin S (SLS) is unrelated to SLO and other cholesterol-binding cytolysins and its functions are associated with intracellular invasion of GAS [241]. Based on these data, the hypothesis of our study was that GAS with impaired ability to produce streptolysins would induce differential immune reponses compared with wt GAS in human macrophages. However, streptolysin deficient bacteria, SLO^- and SLS^-, did not induce differential cytokine responses compared with wild type GAS in human macrophages (Fig. 1 in III). This suggests that streptolysins alone do not contribute to the immune responses induced in human macrophages by GAS stimulation. Instead, multiple virulence factors are involved in the bacterial pathogenicity of GAS and the outcome of inflammatory responses against this bacterium.

A previous report showed that SLO and bacterial viability were essential for caspase-1 activation and IL-1β processing in murine macrophages [242]. These results are conflicting with our data which show similar IL-1β processing in macrophages stimulated with live, inactivated, or streptolysin deficient GAS (Fig. 5 in III). The differences between these studies are likely due to different host species. Of note, mouse is not a natural host for GAS, thus the responses are likely to be

different between murine and human cells. Since human macrophages seem to respond with a quite similar proinflammatory response also to different non-pathogenic bacteria (Fig. 5) it might be typical for macrophages to induce a strong proinflammatory response to any kind of microbial stimulation.

Activation of the NALP3 inflammasome in macrophages is not dependent on streptolysins since wild type GAS as well as virulence factor defective mutants SLO^- and SLS^- induced inflammasome activation equally well (Fig. 5 in III). It has been suggested that bacterial toxins such as listeriolysin O (LLO) and streptococcal streptolysins SLO and SLS are involved in initiating the second signal for the inflammasome activation which leads to caspase-1 activation and maturation of IL-1β [242]. However, this was not supported by our studies and it is possible that some other bacterial or cellular factors in GAS-stimulated macrophages provide the second signal for inflammasome activation. Bacteria-induced cell stress may also be able to activate the inflammasome. Inflammasome activation requires the cooperation of multiple signalling pathways since inhibition of MAPK (ERK, p38, JNK), PI3K, or NF-κB pathways with pharmacological inhibitors resulted in impaired production of mature IL-1β in GAS-stimulated macrophages (Figs. 7 and 8 in III).

Type I IFNs play an important role in host protection against bacteria and viruses [106, 116]. There are several reports on the ability of different bacteria to induce type I IFN production. Intracellular pathogens such as *Salmonella* Typhimurium, *Shigella flexneri* [106, 243] and *L. monocytogenes* [106, 244, 245] as well as some extracellular streptococci [113, 246, 247] and *Lactobacillus* species [51, 212] are able to induce type I IFN responses. Results from Miettinen et al. [212] show that type I IFNs are not induced by all Gram-positive bacteria, since the two *Lactobacillus* strains LGG and LC705 differentially induced type I IFNs. It has been shown previously that GAS-encoded cytolysins SLO and SLS were not required for IFN responses in murine macrophages [191]. These results are in line with ours, showing that IFN production in human macrophages after GAS-stimulation is independent of streptolysins. This is interesting since it was shown previously that cytolysins or their indirect effects are required for type I IFN production in *L. monocytogenes* infection [210, 248-250]. The *Listeria*-derived cytolysin LLO, which is responsible for the IFN-β responses [249], is functionally related to streptococcal SLO. The IFN-β production induced in GAS-stimulated macrophages indicates signalling via internalized TLRs or recognition of the bacterium by cytoplasmic or endosomal receptors.

5.2.6 Role of dynamin-dependent endocytosis in inflammatory responses (III)

Dynamin-dependent endocytosis is known to be involved in the internalization of particles into cells including different macromolecules, bacteria, and viruses [81-83] However, there are endocytic pathways including macropinocytosis which do not require dynamin (Figure 4) [251]. Some internalization pathways are constitutively active while others are triggered only by specific signals. Blocking the functions of dynamin that is involved in pinching of vesicles from the plasma membrane attenuates the internalization of many invasive bacteria with active internalization mechanisms [243, 252, 253]. GAS is mostly considered as an extracellular bacterium without active internalization mechanism and has also the ability to cause invasive infections. It is controversial whether GAS can really enter the cells and multiply in them. Phagosomal escape and limited survival in non-phagocytic [254-256] and phagocytic cells [257, 258] have been reported previously.

At the time this study was started, previous studies had shown that GAS could induce type I IFN production in mouse macrophages in a partly MyD88-dependent manner via activation of IRFs [116, 191]. These reports also showed that in mouse macrophages inhibition of dynamin-dependent endocytosis completely abolished GAS-induced IFN-β production [116]. Type I IFN production is generally thought to result from TLR signalling in endosomal compartments after receptor internalization (TLR2 [259], TLR4 [80]) or after endosomal recognition of components derived from intracellular bacteria or viruses (TLR3, TLR7, TLR8, TLR9). Gratz and coworkers [116] showed that in cells treated with dynasore, a dynamin inhibitor, less internalized bacteria were detected at 2 h after stimulation but equal amounts in dynasore-treated and -non-treated cells at 3 h after stimulation. This suggests that dynasore could slow down the bacterial uptake process. However, inhibition of IFN-β mRNA production was seen up to 8 h, indicating that dynasore affects the events after bacterial internalization. These studies also showed that the ligands that initiate IFN-β production and the signalling molecules involved in these responses were cell type-specific and differed in mouse DCs and macrophages. Since mouse is not a natural host species of GAS, the responses and signalling molecules may be different between mouse and human cells.

Thus, we went on to characterize the role of dynamin-dependent endocytosis in the immune responses induced by GAS in human macrophages. We wanted to find out whether dynamin-dependent endocytosis is responsible for the GAS-induced IFN-β production in human macrophages. Inactivated GAS was included in the studies since it was assumed not to be able to deliver GAS-derived components into the host cell cytosol. GAS mutants defective in streptolysin genes SLO and SLS were also

included, because SLO has been shown to mediate the delivery of GAS-derived molecules into the host cytosol and promote bacterial escape from phagosomes into the cytosol [239, 260, 260] and SLS is believed to be associated with intracellular invasion of GAS [241]. Our studies show that inhibition of dynamin-dependent endocytosis in human macrophages by dynasore almost completely blocked cytokine mRNA expression, including IL-1β, TNF-α, IFN-β, and CXCL-10 (Figs. 2 and 3 in III). These results suggest that functional dynamin is required for the activation of signalling cascades that lead to cytokine gene expression after GAS-stimulation. It is well known that signalling from the cell surface TLRs leads to the activation of NF-κB and MAPK pathways resulting in the production of proinflammatory cytokines, such as TNF-α and IL-1β. Endosomal TLRs in turn trigger both IRF-mediated IFN-β and NF-κB-mediated cytokine gene expression. TLR2 and TLR4 internalization after ligand binding are dynamin-dependent events [53, 80], which is likely to explain at least partly the disturbed IFN-β expression by dynasore treatment. Brandt et al. [53] have also suggested that TLR2-dependent NF-κB activation requires receptor internalization in human monocytes. They used TLR ligands in the experiments, and the responses induced by whole bacteria may differ from those induced by ligands. Nevertheless, Gratz et al. [191] excluded the involvement of TLR2, 4, and 9, and later on the role of TLR3 and 7 [116] in GAS-induced IFN-β responses in mice and suggested that a new cytoplasmic MyD88-dependent receptor is involved. However, the putative novel molecule has remained uncharacterized.

Since, proinflammatory cytokines TNF-α and IL-1β were blocked by dynasore treatment (Fig. 2 in III) it seems that also signalling from the cell surface and the activation of the inflammasome complex are inhibited. It was previously shown that some inflammatory responses including cytokine production do not necessarily require internalization of the target [77-80, 261]. However, also publications supporting receptor internalization exist [53, 259]. The inconsistencies between these studies may be due to different methods used. The use of mouse macrophages and DCs, cell lines, and different stimulants such as microbial components, whole bacteria, and viruses are likely to contribute to the results. It remains unclear whether receptor internalization is required for signalling.

Dynamin is involved in the internalization of cytokine receptor chains and inhibition of these pathways decreased STAT translocation into the nucleus [83, 84]. Thus, dynasore treatment may interfere with cytokine networks and their positive and negative feedback loops. Sendai virus which is able to directly fuse with the plasma membrane [179] and primarily does not use dynamin-dependent mechanisms for internalization efficiently induced IFN-β expression that was affected by dynasore to a lesser extent (Fig. S2 in III). Interferon-inducible CXCL10 was also induced by

Sendai virus, but its expression was in contrast to IFN-β attenuated by dynasore. Thus, at least IFN-β mRNA expression and secretion induced by intracellular ligands were not completely inhibited by dynasore, since IFN-inducible effects were seen. Based on this data, it could be hypothesized that dynasore is able to interfere with dynamin-dependent signal transduction from the cell surface, resulting in impaired cytokine responses but unable to interfere with IFN-β expression induced by intracellular ligands. Dynamin is also involved in actin dynamics and inhibition by dynasore possibly interferes with vesicle formation and protein transport inside the cells [262, 263]. Data concerning the involvement of dynamin in different cellular events is contradictory, since some of these studies are done in yeast instead of mammalian cells and the effects may be quite different between non-phagocytic and phagocytic cells. We conclude that dynamin is required in the induction of cytokine mRNA expression in human macrophages in response to GAS-stimulation. However, the exact mechanism remains unexplained.

6 Concluding remarks

In recent years, the knowledge of the interactions between bacteria and their hosts has emerged considerably. There is a growing interest towards the health promoting properties of probiotic bacteria in the developed world with an increasing incidence in asthma and allergy-related diseases. Conclusive evidence is still lacking on the mechanisms of probiotic actions because a wide range of different doses and bacterial strains are used in various animal and cell models in probiotic studies. The mechanisms behind the observed clinical effects of probiotic bacteria as well as the mechanisms of bacterial infections remain largely unexplained and the pathogenic group A streptococcus, GAS, is among the ten most common causes of death from infectious diseases despite effective antibiotic treatment.

The initial aim of the present study was to analyse the ability of potentially probiotic bacterial strains to induce cytokine responses in comparison to each other and to the pathogenic bacterium GAS in order to facilitate the selection of new bacteria to probiotic clinical trials. Pathogenic GAS was analysed to reveal the contribution of streptococcal virulence factors, streptolysins, in the pathogenicity of the bacterium and the role of dynamin-dependent endocytosis in these responses.

Human monocyte-derived macrophages and DCs were used in these studies, since in addition to epithelial cells they are likely to interact with commensal, pathogenic, and probiotic bacteria *in vivo*. Various bacterial strains exhibited different capacities to induce innate cytokine responses in human macrophages and DCs. Macrophages responded quite similarly to non-pathogenic and pathogenic bacteria with strong proinflammatory cytokine responses. Most of the bacteria were also potent inducers of anti-inflammatory IL-10 in macrophages. We found that non-pathogenic bacteria were able to induce DC maturation as efficiently as the pathogenic GAS. Cytokine production in DCs showed more variation between analysed bacteria, indicating a more selective role of DCs in initiating immune responses towards non-pathogenic and pathogenic bacteria. The main findings were that non-pathogenic bacteria were able to induce DC maturation and cytokine production in both cell types, some even more efficiently than pathogenic GAS.

The other main focus of this work was to clarify the significance of bacterial viability and the role of streptococcal virulence factors in immune responses. We found that there were no significant differences in the ability of live, inactivated or streptolysin deficient bacteria to induce cytokine responses. Thus, secreted bacterial

components are probably not the major regulators of cytokine responses. Cytokine responses are obviously regulated by multiple intracellular signalling pathways in macrophages and DCs with SOCS3 playing a role at least in IL-10 mediated immune responses in macrophages. We also found out that dynamin-dependent mechanisms were involved in bacteria-induced inflammatory responses. It is likely that these responses result from the cooperation of multiple receptors and receptor complexes activated at the cell surface as well as intracellularly. This leads to the activation of MAPK, NF-κB, PI3K, and IRF pathways which in case of disturbance in one pathway most likely compensate each other in order to maintain the capacity to initiate appropriate innate immune responses.

Results of these studies emphasize the complex interactions between microbes and host cells and inspire to continue research to reveal the molecular mechanisms involved in biological actions of probiotic bacteria. It is also important to understand the complexity and mechanisms of the immune responses induced by pathogenic microbes in order to control tissue destruction associated with inflammatory responses induced by pathogenic bacteria. This information may be useful in further development of novel therapeutic strategies against infectious diseases for example by modulating the magnitude and quality of cytokine responses. In addition, the cytokine production profiles induced by non-pathogenic bacteria could help in selecting new potential probiotic bacteria for further studies and for clinical use.

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