Regulation of innate and adaptive immune responses by Gram-positive and Gram-negative bacteria

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ABSTRACT

Bacteria are classified as Gram-positive or Gram-negative, depending on their cell wall structure. The role of the bacterial cell wall in immune regulation is the focus of the current work.

Most Gram-positive bacteria stimulate monocytes to produce large amounts of IL-12. IL-12 induces production of IFN-γ in T cells and NK cells, which, in turn, activates the bactericidal capacity of the phagocyte in synergy/concert with TNF, produced by macrophages. We studied the bacterial structures and signalling pathways involved in IL-12 production in response to intact Gram-positive bacteria. This production depended on phagocytosis and activation of the JNK, NF-κB and PI3K pathways. Gram-positive bacterial fragments inhibited IL-12 production, which may serve as a negative feedback to turn off phagocyte activation when the bacteria have been destroyed.

S. pneumoniae is a Gram-positive pathogen with a peculiar habit to disintegrate in stationary culture, due to activation of autolytic enzymes that degrade the cell wall. We demonstrated that pneumococci undergoing autolysis generate bacterial fragments that shut off monocyte production of TNF, IFN-γ and IL-12, thereby counteracting phagocyte activation. Further, the cytoplasmic pneumococcal toxin pneumolysin that was released upon autolysis dramatically augmented radical oxygen production in human neutrophils. Notably, ROS were foremost produced into intracellular compartments, probably affecting neutrophil function.

We also studied differences in how Gram-positive and Gram-negative bacteria modulate presentation of a model antigen to naïve T cells. Different subsets of mouse antigen-presenting cells were fed soluble ovalbumin (OVA), or OVA produced inside transgenic Gram-positive (lactobacilli/lactococci) or Gram-negative (*E. coli*) bacteria. Proliferation and cytokine production by OVA-specific transgenic T cells (DO11.10) was used as read-out system. “Bacterial” OVA much more efficiently activated OVA-specific $CD4^+$ T cells, than did soluble OVA. Further, *E. coli*-OVA induced a greater T cell proliferation than did OVA expressed by Gram-positive bacteria. Splenic APCs pulsed with soluble OVA induced IL-13 production, while *E. coli*-OVA induced both IFN-γ and IL-13 and lactobacilli-OVA induced a weak IFN-γ response in the T cell culture. We also noted that peritoneal DCs induced a different T cell polarisation pattern compared to splenic DCs, supporting production of more IL-17 and IL-10, but less IL-13. Furthermore, the presence of peritoneal macrophages inhibited $CD4^+$ T cell activation to bacterial, but not to soluble, antigens.

Key words: Gram-positive, Gram-negative bacteria, *Streptococcus pneumoniae*, IL-12, autolysin, pneumolysin, monocytes/macrophages, dendritic cells, $CD4^+$ T cells

ORIGINAL PAPERS

This thesis is based on the following papers, which are referred to in the text by their Roman number (I-IV):

I. Cecilia Barkman, Anna Martner, Christina Hessle and Agnes E. Wold. 2008. **Soluble bacterial constituents down-regulate secretion of IL-12 in response to intact Gram-positive bacteria.** *Microbes and infection* 10:1484-93.

II. Anna Martner, Susann Skovbjerg, James C. Paton, Agnes E. Wold. **Autolysis of *Streptococcus pneumoniae* prevents phagocytosis and production of phagocyte activating cytokines.** *Submitted.*

III. Anna Martner, Claes Dahlgren, James C. Paton and Agnes E. Wold. 2008. **Pneumolysin released during *Streptococcus pneumoniae* autolysis is a potent activator of intracellular oxygen radical production in neutrophils.** *Infection and immunity* 76:4079-4087.

IV. Anna Martner, Sofia Östman, Samuel Lundin, Lars Axelsson and Agnes E. Wold. **Gram-negative bacteria are superior $CD4^+$ T cell activators compared to Gram-positive bacteria.** *In manuscript*.

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ABBREVIATIONS

APC	Antigen-presenting cell
cDC	Conventional dendritic cell
DC	Dendritic cell
IFN	Interferon
IL	Interleukin
IRAK	IL-1 receptor associated kinase
JNK	C-jun N-terminal kinase
LPS	Lipopolysaccharide
LTA	Lipotechoic acid
MAL	MyD88-adaptor like
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
MyD88	Myeloid differentiation factor 88
NF-κB	Nuclear factor κ B
NLR	Nod-like receptor
NOD	Nucleotide-binding oligomerisation domain
OVA	Ovalbumin
P38	Protein 38
PBMC	Peripheral blood mononuclear cells
pDC	Plasmacytoid DC
TCR	T cell receptor
TGF-β	Transforming growth factor β
Th	T helper
TIRAP	TIR domain-containing adaptor protein
TLR	Toll like receptor
TNF	Tumour necrosis factor
TRAM	TRIF related adaptor molecule
TRIF	TIR domain-containing adaptor inducing IFN-β

INTRODUCTION

The innate and adaptive immune systems cooperate to defend the integrity of the body against the constant threat of microbes. Without a defence system, the bacteria that colonise the skin and mucosal membranes would invade us. Other microbes have evolved strategies to circumvent immune defence and can cause disease even in individuals with a perfectly functional immune system. These are termed pathogens and the factors that enable them to cause disease are termed virulence factors. In this thesis, innate and adaptive immune responses to commensal bacteria and a selected pathogen, *S. pneumoniae* (the pneumococcus) are studied.

Cell wall structures of Gram-positive and Gram-negative bacteria

The most common way to classify bacteria is according to their Gram-staining properties. Bacteria are stained with crystal violet, fixed with an iodide solution, destained with ethanol or acetone, and counterstained with safranin. Gram-positive bacteria retain crystal violet complexes in the cytoplasm, while they readily leak out of Gram-negative bacteria which are decolourised (1, 2) (Fig. 1). The difference in staining properties relates to the cell wall composition of Gram-positive and Gram-negative bacteria, respectively (Fig. 2).

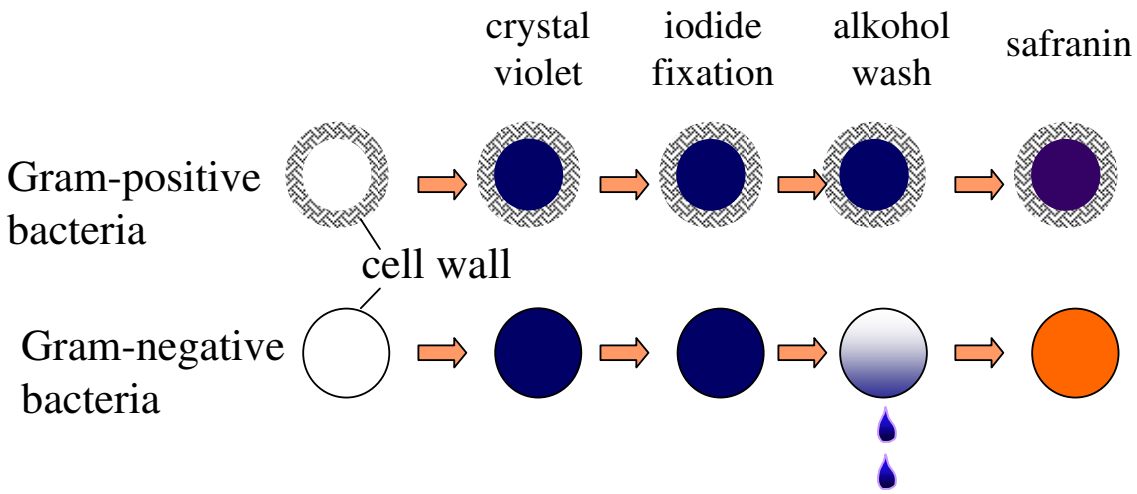

Figure 1. Gram-positive bacteria are stained blue, while Gram-negative bacteria become red upon Gram-staining.

The Gram-positive cell wall

The cell wall of Gram-positive bacteria is composed of a thick homogenous layer of peptidoglycan, which is a polymer of the sugars N-acetyl-glucosamine and N-acetyl-muramic acid linked together by peptide inter-bridges. The crystal violet-complexes are entrapped by these cell wall meshes.

Most Gram-positive cell walls contain teichoic and lipoteichoic acid which, when present, are vital for survival of the bacteria (3). Teichoic acids are long negatively charged polymers of glycerol or ribitol phosphates, covalently linked to peptidoglycan. Lipoteichoic acid (LTA) consists of a polymer of glycerol phosphate linked to a glycolipid inserted in the cytoplasmic membrane. LTA may be shed from the membrane during bacterial growth.

More recently, the Gram-positive cell wall was recognised to also contain lipoproteins. These are anchored to the bacterial membrane and may also may be covalently linked to the peptidoglycan layer, LTA or teichoic acids (4). The lipoproteins might be dispensable for growth of Gram-positive bacteria, but mutants generally show reduced virulence (5). (Fig. 2)

The Gram-negative cell wall

The peptidoglycan layer in the cell wall of Gram-negative bacteria is thin and unable to retain the crystal violet-protein complexes. Furthermore, the third amino acid of the peptide chain that links the sugar polymers is diaminopimelinacid (DAP) in Gram-negative bacteria, while Gram-positives normally have L-lysine in this position (6).

Gram-negatives have an outer membrane containing lipopolysaccharide (LPS) and lipoproteins (3). LPS is unique to Gram-negative bacteria, and is the most potent inflammatory molecule existing, inducing strong inflammatory reactions in humans in minimal doses (7-9). LPS consists of a lipid A domain, composed of phosphate-linked glucosamine disaccharides with bound fatty acids attaching it to the outer membrane and attached strain-specific oligosaccharide chains (O-antigen). The lipid A part is vital for survival of the Gram-negative bacteria, while the O-antigen is not necessary for bacterial function. LPS is shed during bacterial growth.

Lipoproteins are present in the outer membrane of Gram-negatives; either freely associated to the membrane, or covalently attached to the peptidoglycan layer anchored it to the outer membrane. In Gram-negatives, lipoproteins are essential for bacterial survival (4, 5, 10, 11). (Fig. 2)

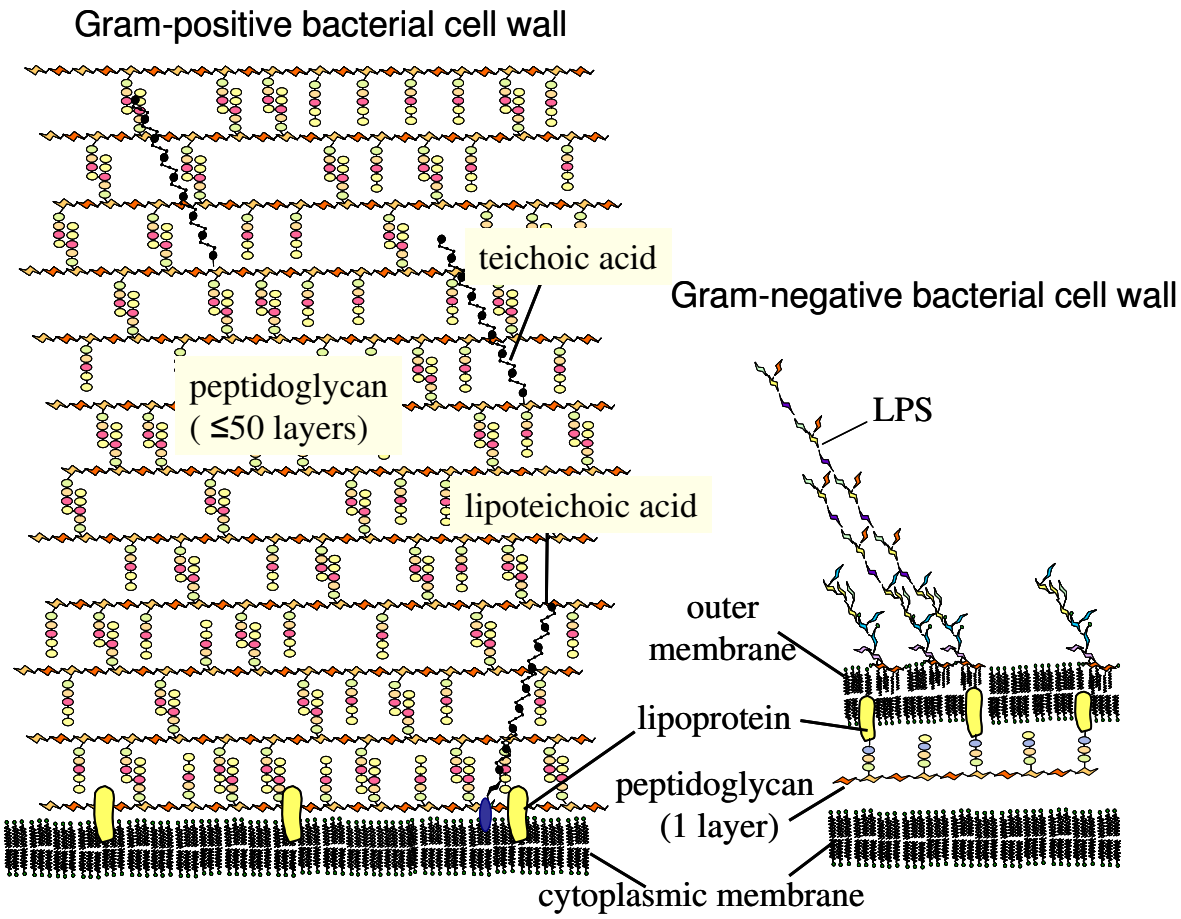

Figure 2. Composition of the Gram-positive and Gram-negative bacterial cell walls, adapted from Mölne and Wold, 2007.

Innate recognition of microbes

For appropriate host defence, microbes must be recognised and destroyed by our immune system. The innate immune system consists of cells and complement that are activated by conserved microbial structures. Cells in the innate immune system, such as monocytes, macrophages, dendritic cells and neutrophils, express a variety of receptors enabling them to recognise these microbial structures, so called pattern recognition receptors (12-14).

Family	Receptor	Adaptor	Ligand
TLR	TLR2	TIRAP/MyD88	Lipoproteins, LTA?, PG?
	TLR4	TIRAP/MyD88 TRIF/TRAM	LPS, pneumolysin
	TLR5	MyD88	Flagella
	TLR9	MyD88	Unmethylated CpG-DNA motifs
NLR	NOD1	RIP2	Meso-DAP in Gram-negative PG
	NOD2	RIP2	Muramyl-dipeptide in PG
C-type lectin receptors	Mannose receptor		Mannose, fucose > N-acetylglucosamin > glucose
	DC-SIGN		Mannose, fucose
Complement receptors	CR3 = CD11b/CD18		C3b, iC3b
	CR4= CD11c/CD18		C3b, iC3b

Table 1. Examples of receptors recognising bacteria

Phagocytic receptors

Certain pattern recognition receptors promote uptake of microbes into phagsomes. These phagocytic receptors are expressed by macrophages and other phagocytic cells and include scavenger and C-type lectin receptors, as well as receptors recognizing complement and other opsonins.

Scavenger receptors

Scavenger receptors are abundantly expressed on macrophages, and may also be expressed on dendritic cells. They mediate phagocytosis of microbes, as well as necrotic and apoptotic cells. Scavenger receptors recognize large negatively charged molecules such as phospholipids, LPS and teichoic acid and certain bacterial surface proteins (15, 16).

C-type lectin receptors

The C-type lectin receptors (CLRs) recognise specific sugar structures that are present in microorganisms, but normally absent in healthy mammalian glycoproteins, though they may appear on ageing self-glycoproteins. Macrophages and dendritic cells express a variety of CLRs, including the mannose-receptor and DC-SIGN (17). In DCs, antigens that are taken up via CLRs become presented on

MHC I and MHC II. In addition to mediating phagocytosis, certain CLRs, such as dectin-1 and DC-SIGN, have been shown to modulate TLR-signalling (18-20).

Opsonin receptors

Opsonisation of microbes with complement or antibodies greatly enhances their phagocytosis. Fcγ-receptors, expressed by macrophages, neutrophils and DCs, bind to the Fc-part of IgG antibodies, facilitating uptake of antibody-targeted microbes or immune complexes (21). Complement activation results in deposition of iC3b on microbial surfaces. Bacteria opsonised with iC3b are recognised and phagocytosed by innate immune cells expressing complement receptor (CR3) (CD11b/CD18) and CR4 (CD11c/CD18) (22). In human CR3 is highly expressed on circulating monocytes and neutrophils, but for tissue macrophages CR4 appears to be the most abundant receptor for iC3b (23). In mice, CD11b is expressed in high levels by macrophages, monocytes, neutrophils and certain DC-subsets, while CD11c is considered to be a DC marker. The role for CD11c in antigen-presentation remains ill characterised, but expression of CD11c on cell types not regarded as typical DCs has been coupled to increased antigen-presenting capacities (24-26).

Toll-like receptors

A group of mammalian receptor proteins, homologous to the drosophila Toll proteins, have been given the name Toll-like receptors (TLRs). Signalling through TLRs is important to alert the immune system of potential danger. The TLRs recognise conserved molecule structures that normally are essential for survival of the microbe, an elegant design to prevent microbes from escaping innate recognition. So far, 10 TLRs have been identified in humans and 13 TLRs in mice (12). The TLRs involved in recognition of bacteria include TLR2, TLR4, TLR5 and TLR9 and are listed in table 1. TLR2, TLR4 and TLR5 recognise bacterial cell wall components or flagella and are located at the cell surface of phagocytic cells, but may also be recruited into phagosomes (27, 28). In contrast, TLR9, which recognises bacterial DNA, is only expressed intracellularly (29-31).

TLR2

TLR2 has been suggested to recognise a wide range of microbial products, including lipoproteins, LTA, peptidoglycan and certain types of LPS. It is clear that lipoproteins from both Gram-positive and Gram-negative organisms are recognised by this receptor (32, 33), but lipoproteins contaminating LTA and PG preparations may be responsible for the stimulatory effects of these substances (34-38). TLR2 generally functions as a heterodimer in combination with either TLR1 or TLR6, where the TLR2/TLR1 heterodimer recognises triacylated lipoproteins and the TLR2/TLR6 heteromers recognises diacylated lipoproteins (32, 39, 40).

Furthermore, co-receptors such as CD36 or CD14 may be involved in optimal activation of TLR2 (41, 42).

Though structures in both Gram-positive and Gram-negative bacteria may be recognised by TLR2, most studies support a predominant role for TLR2 in recognition and host defence to Gram-positive organisms (43-46). Perhaps TLR2 stimulatory structures are more exposed on the surface of Gram-positive, than Gram-negative, bacteria. Indeed, studies on the major lipoprotein in *E. coli*, the Braun lipoprotein, have shown that it is immunogenic and antigenic only in cells exhibiting an abnormal outer membrane structure, indicating that it normally is not exposed on the bacterial cell surface (47). Signalling through TLR2 is initiated by recruitment of TIRAP, which in turn recruits the adaptor protein MyD88 (48) (Fig. 3).

TLR4

LPS, which is exclusively expressed by Gram-negative bacteria, activates TLR4. LPS forms a complex with LPS-binding protein, which is recognised by CD14 on the macrophage surface. CD14 in turn binds to TLR4 via the co-receptor MD-2 (49-51). TLR4 stimulation leads to activation of two distinct intracellular signalling pathways. Similarly to TLR2, MyD88-dependent signalling is initiated after recruitment of TIRAP (48, 52), but in addition TRIF dependent signalling is initiated after recruitment of TRAM (53, 54) (Fig. 3). The fact that LPS activates both MyD88 and TRIF may be the reason for the superior inflammatory activating potential of LPS. In addition to LPS, certain other bacterial molecules, such as the *Streptococcus pneumonia* toxin pneumolysin has been shown to signal via TLR4 (55, 56).

Expression of TLRs

TLRs are widely expressed in many cell types, including epithelial and endothelial cells, although these non-hematopoietic cell types normally express only selected subsets of TLRs (12, 57). In contrast, hemotopoetically derived cells, such as monocytes, macrophages, neutrophils and dendritic cells (DCs), generally express a wider spectrum of TLRs (12, 57). The TLR expression is not static, but may be up- or down regulated by various stimuli and factors present in the tissue (58-60).

Expression of TLRs may vary between species. Only mouse, but not human, conventional DCs (cDC), express TLR9, while both mouse and human plasmacytoid DCs and B cells strongly express this receptor (61-63). Furthermore, murine cDCs from the intestinal *lamina propria* express TLR9, but not TLR4, while cDCs from the spleen express both TLR4 and TLR9 (64). In contrast, *lamina propria* cDCs in humans express TLR4, but not TLR9 (58, 60). Care should be taken when interpreting data of CpG-DNA stimulation in mice concerning the relevance for the human host.

NOD-like receptors

Another family of receptors, important in signalling danger to the immune system, is called the NOD-like receptors (NLRs). The NLRs may react to both microbial and endogenous danger factors. They are cytosolic proteins probably sensing their ligands when present inside the cell (65). Though the family has at least 20 members, the ligands have only been identified for a few of the receptors (66-68). The NLRs have C-terminal leucin-rich repeats (LRR), directly or indirectly responsible for ligand binding, a centrally localised NACHT domain that facilitates self-oligomerisation after activation, and variable N-terminal recruiting domains, that recruits adaptor proteins upon oligomerisation. The recruiting domains of NOD1 and NOD2 are called CARDs, NOD1 possessing one and NOD2 two CARD-domains (69).

NOD1

NOD1 detects the amino acid DAP, in Gram-negative bacterial peptidoglycan (66, 67). Among the Gram-positive species, only peptidoglycan from *Listeria* and *Bacillus* are known to contain this amino acid (66). Binding to NOD1 leads to exposure of CARD-domains and recruitment of the adaptor protein RIP2, which in turn stimulates TAK1. This leads to IKK dependent activation of NF-κB and activation of MAPK (70, 71) (Fig. 3).

NOD2

NOD2 binds to muramyl dipeptide (MDP), the basic building block of both Gram-positive and Gram-negative peptidoglycan (68). Polymerisation results in RIP2 recruitment, NF-κB and MAPK activation (70, 71) (Fig. 3). In addition, NOD2, but not NOD1, was recently shown to directly interact also with NALP3 (72).

NALP3

NALP3 is activated by danger associated host factors, such as uric acid crystals (73). Upon activation it polymerizes into an inflammasome, that recruits caspase 1, leading to cleavage of pro-IL-1 β and subsequent release of IL-1β.

Expression of NODs

NOD1 and NOD2 are the most studied members of the NLR family. While NOD1 is ubiquitously expressed, NOD2 appears in monocytes, macrophages, dendritic cells and epithelial cells (65, 74-76). Mutations in especially NOD2, but also NOD1, are enriched in patients with the inflammatory bowel disease Crohn´s disease (77-79). Furthermore, NOD2-/- mice are more susceptible to infections with *S. aureus* (80).

Signalling through pattern recognition receptors

When innate immune cells recognise bacteria via their TLRs and NLRs, signalling cascades are induced leading to synthesis and release of soluble mediators or expression of surface molecules that contribute to initiating an inflammatory response and a subsequent specific immune response.

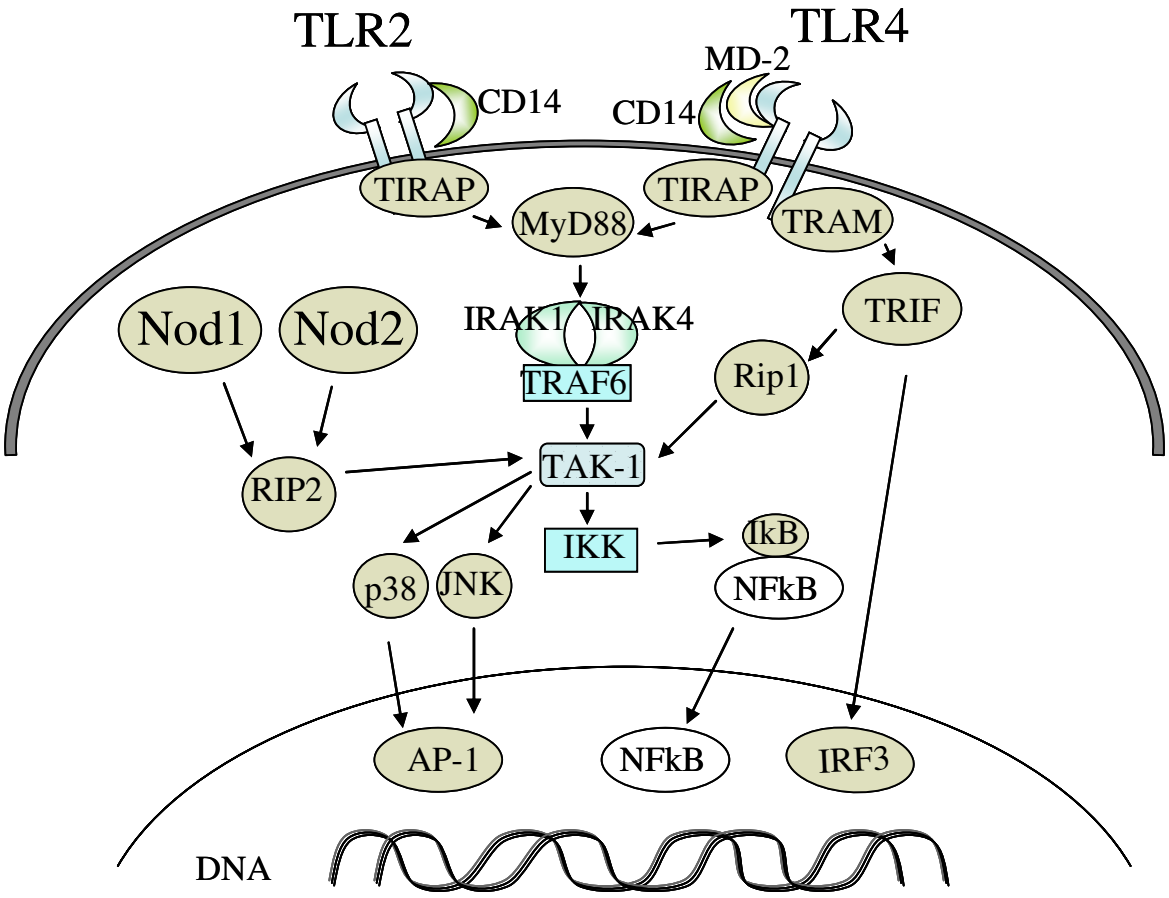

Figure 3. Intracellular signalling pathways induced by activation of TLR2, TLR4, NOD1 and NOD2.

TLR signalling

Since TLRs have no proper signalling domain, they rely on adaptor proteins for convening downstream signals. These include MyD88, TIRAP, TRIF, TRAM and SRAM. Different usage of these adaptor proteins, various subcellular localisation of TLRs and the ability to signal simultaneously via multiple TLRs or TLRs

together with other receptors, permit different types of cellular responses to be induced (81).

The MyD88 adaptor protein is used by all TLRs except TLR3 and MyD88-/- mice have increased mortality in infection models (82, 83). MyD88 recruits and phosphorylates members of the IRAK family, out of which IRAK4 is indispensable for further activation. Downstream, TAK-1 is activated, which stimulates IKK-dependent activation of the transcription factor NF-κB, as well as MAP-kinase dependent activation of the AP-1 transcription factor (Fig. 3). As a result, genes encoding inflammatory enzymes and cytokines are transcribed (81). Patents with mutations in IRAK4 are highly susceptible to infections by foremost the Gram-positive bacteria *Streptococcus pneumoniae* and *Staphylococcus aureus*. Their inflammatory responses are delayed and they sometimes die from their infections (84).

Signalling via TRIF is specific for TLR4 and TLR3. Thus, TLR4 is the only TLR that can stimulate both the MyD88- and TRIF-dependent signalling pathways. Signalling via TRIF activates the transcription factor IRF-3 and induces transcription of IFN-β and IFN inducible genes (81). In addition, TAK-1 can also be activated. Hence, when cells from MyD88-deficient mice are stimulated with TLR4 ligands, the NF-κB and MAP-kinases are activated via TRIF, but the production of inflammatory cytokines is slower and reduced in magnitude (85).

As also NOD activates TAK-1 to induce activation of NF-κB and the MAP-kinases, signals via the TLRs and NODs converge on TAK-1. Thus activation of TLRs and NODs may act synergistically to induce production of proinflammatory cytokines (86, 87).

Pathways negatively regulating PRR signalling

Excessive immune and inflammatory activation is dangerous and appropriate regulation of the TLR and NLR signalling events is crucial to prevent shock, tissue damage and organ failure. Thus, pathways, including PI3K, MKP-1 and SOCS that inhibit inflammatory cytokine production are induced upon TLR activation and function as negative feedback mechanisms.

PI3K – multiple positive and negative effects via different pathways

The PI3K pathways can be activated via stimulation of TLRs, NODs and growth factor receptors and regulate many processes in the cell, such as cell growth, proliferation, phagocytosis and phagosome maturation (88-90). In addition, PI3K has been reported to negatively regulate IL-12 production in response to soluble ligands of TLR2, TLR4 and NOD2 in monocytes, macrophages and cDCs (91-95). The mechanism of inhibition is though to involve activation of the mammalian target of rapamycin (mTOR) and inhibition of glycogen synthase kinase 3 (GSK3) (95) (Figure 4).

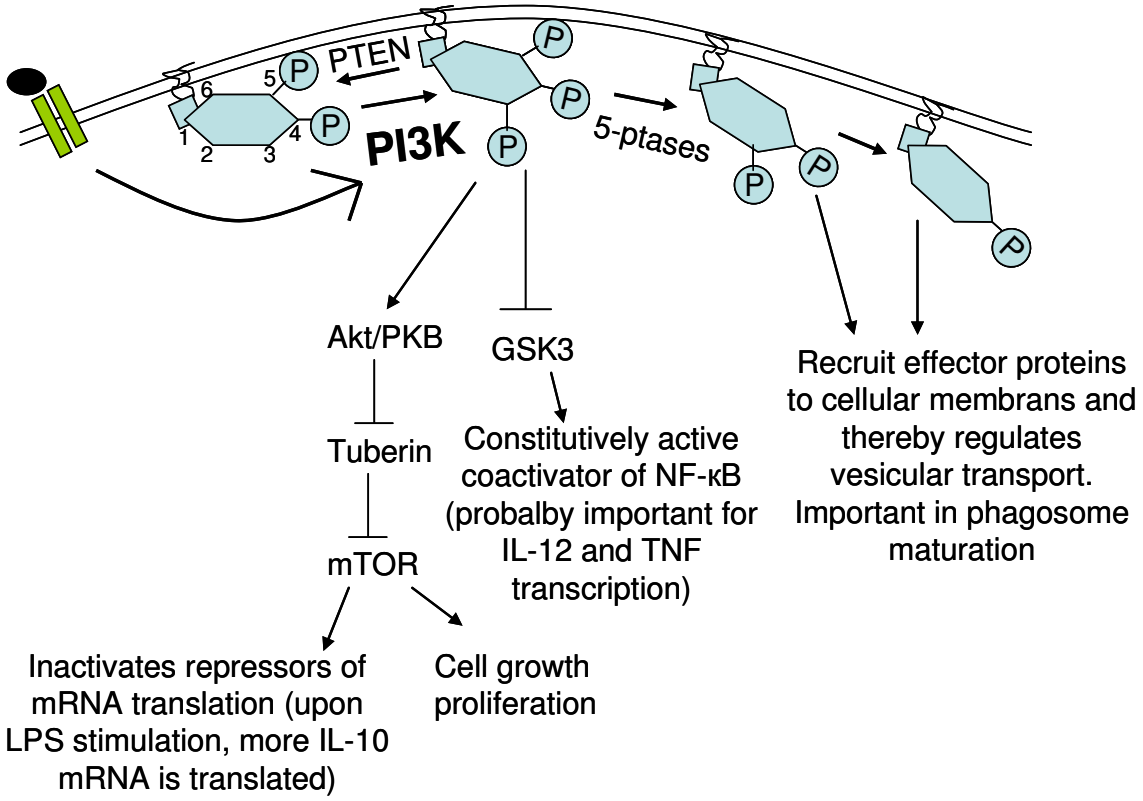

Figure 4. Binding to TLRs, NODs and growth factor receptors activates tyrosin kinase adaptor molecules on the membrane, which recruit PI3K. PI3K phosphorylates membrane-bound phophatidylinositols into PtdIns(3,4,5)P_3. The PtdIns(3,4,5)P_3 can activate Akt/PKB and inactive GSK3, but is short-lived and PTEN and 5-phosphatases compete for dephosphorylation at different positions of the inositol ring. By the action of 5-phosphatases, membrane bound substrates are formed that can recruit around 300 different proteins and thereby regulate vesicular transport. These PtdIns variants are mainly, but not exclusively, formed at intracellular membranes (95-97) .

MKP-1

The mitogen-activated protein kinase (MAPK) phosphatase-1 (MKP-1) is a phosphathase that preferably dephosphorylates p38 and JNK, leading to their inactivation and, as a result, reduced cytokine production to TLR ligands (98).

SOCS

The suppressors of cytokine signalling (SOCS) proteins have been suggested to interfere with TLR signalling, although their major function probably is to inhibit the JAK/STAT signalling transduction and thereby prevent cytokine signalling (99).

Feedback systems in different cell types

The relative importance of inhibitory pathways may differ with cell type and activation state of the cell. For example, treatment of myeloid cells with IL-10, diminishes the ability of TLR and NOD ligands to induce NF-κB activation (100), whereas IFN-γ activated cells are less affected by several of the inhibitory mechanisms on TLR signalling (101).

To avoid septic shock, it is of particular importance to have effective feedback systems in cells circulating in the blood. For example, in infection models, mice prevented from signalling via the PI3K were more susceptible to dying of septic shock (102). Monocytes are poor producers of IL-12 in response to Gram-negative bacteria and bacterial components such as LPS (103). Upon differentiation to dendritic cells they more readily produce IL-12 in response to Gram-negative bacteria and bacterial components (59). A possible explanation could be that inhibitory pathways triggered by TLRs are easier to activate in monocytes, than in dendritic cells.

Though several studies have shown a synergism in TLR and NOD signalling for production of proinflammatory cytokines (86, 87), there are also reports of the opposite (104, 105), indicating that the negative feedback pathways induced via TLRs via NODs inhibit each other.

Phagocytosis and intracellular killing of bacteria

Phagocytosis is, together with the complement system, the most important antimicrobial system in our body. The professional phagocytes include monocytes, macrophages and neutrophils. The cell wall structure of Gram-positive and Gram-negative bacteria make them dependent on partly different killing mechanisms.

Monocytes/macrophages

Monocytes are phagocytic cells circulating in the blood stream. Upon entering the tissue the differentiate into macrophages or, as it recently has been shown, into tissue dendritic cells (106, 107). Tissue macrophages are heterogeneous and depending on the tissue in which they reside, they have distinct functions and display different patterns of surface molecules. Examples of specialized macrophages include the Kupffer cells in the liver, microglia in the central nervous system, metallophilic and marginal zone macrophages in the spleen, osteoclasts in bone and alveolar and peritoneal macrophages (108, 109). Macrophages produce large quantities of cytokines and are efficient at phagocytosis and killing microbes (108-110).

Recruitment of neutrophils

Tissue resident macrophages provide the first line of defence against invading pathogens. Upon interaction with microbes, macrophages produce an array of mediators, including prostaglandins, NO, TNF, IL-1, IL-8 and MCP-1 that are important in the process of recruiting more immune cells to the infected area that can participate in eliminating the microbe. Prostaglandins and NO dilate the blood vessels and slow down the blood stream, whereas TNF and IL-1 upregulate the adhesion molecules E-selectin and ICAM-1 on the endothelium. The chemokines IL-8 and MCP-1 attract neutrophils and monocytes, respectively (111).

Generalized symptoms of inflammation

Cytokines produced by macrophages in response to microbial activation stimulate the acute phase reaction in the liver. IL-6, TNF and IL-β induce fever and cortical production. TNF and IL-β promote production of neutrophils in the bone marrow and induce alexia and fatigue.

Phagocytosis and Intracellular killing

Phagocytosed bacteria end up in a phagosome. In a process referred to as phagosome maturation, vesicles from the endoplasm tic reticulum or other intracellular compartments merge with the phagosomes, equipping it with a machinery to kill and digest microbes. In macrophages the phagosomes are rapidly acidified and liposomal proteases are recruited, resulting in a highly derivative environment. Further, the inducible nitric oxide synthase is activated in response to IFN-γ and NF-κB activation generating NO to the phagosome, and the NADPH-oxidase is formed, which generates ROS, but in fairly low levels (112). NO and ROS are highly reactive and participate in killing microbes (113). Although TLRs do not trigger phagocytosis, TLR signalling has been shown to either promote or inhibit phagosome maturation, presumably via the PI3K pathways (114, 115).

Phagocyte activation

Bacterial killing inside phagosomes can be enhanced by the action of phagocyte activating cytokines, where IFN-γ and TNF are the key players. TNF is produced by macrophages stimulated with bacteria, while IFN-γ is produced by NK cells and T cells upon activation with IL-12, produced by macrophages. TNF and IFN-γ enhance phagocyte bactericidal mechanisms via activation of iNOS and enhancement of phagosome maturation (116, 117). It is since long recognized that production of IL-12, TNF and IFN- γ is fundamental in host defence against intracellular bacteria (118-120). More recently, animal studies have demonstrated that these phagocyte activating cytokines are important also in defence against extracellular Gram-positive bacteria such as *S. aureus* (121), group B streptococci (45, 122) and *S. pneumoniae* (123-126). Evidently, activation of phagocytic killing mechanisms is needed to defeat these pathogens.

In freshly isolated human monocytes, Gram-positive bacteria induce much more IL-12 and TNF than do Gram-negative bacteria, while the latter induce more IL-10 and PGE_2 than do Gram-positive bacteria (103, 127, 128). Since IL-12 enhances IFN-γ production from NK cells and T cells (129-131), while IL-10 and PGE_2 inhibits the same (132-135), unfractionated PBMC produce much more IFN-γ in response to Gram-positive than in response to Gram-negative bacteria (103, 136). It may be of functional relevance for phagocytes to produce high levels of phagocyte activating cytokines in response to Gram-positive bacteria, in order to achieve digestion of the thick and tightly meshed Gram-positive peptidoglycan cell wall. Gram-negative bacteria, in contrast, may readily be killed by disruption of the outer membrane by defensins and BPI.

Neutrophils

Neutrophils are terminally differentiated phagocytes, with a short life-span, circulating in the blood. During certain inflammatory reactions they accumulate rapidly and in high numbers and play a dominant role in the phagocytosis and killing of microbes. Neutrophils produce only moderate amounts of cytokines, mainly IL-8, but produce also lipid-derived products, such as prostaglandins and leukotrines, and release contents of granules, including toxic metabolites.

Granula

The cytoplasm of neutrophils is densely packed with granula. Four types of granula exist; the specific granula, azurophilic granula, gelatinase granula and secretory vesicles.

The specific granula are most frequent and contain the iron-binding protein lactoferrin as well as lysozyme that degrade peptidoglycan. Further, the membrane

bound part of the NADPH-oxidase, cytochrome b, is situated in the specific granula. The azurophilic granula contains the enzyme myeloperoxidase (MPO), that catalyses production of hypocloric acid from oxygen radicals. It also contains defensins. Gelatinase granules contain metalloproteases, which are enzymes that degrade tissue matrix and facilitate migration of the neutrophils through the tissue. The secretory vesicles contain complement receptors and integrins that rapidly can be mobilised to the cell surface upon cell activation.

Intracellular killing

The concept of phagocytosis and phagosome maturation is valid for neutrophils, as well as macrophages, though the bactericidal content of the formed phagosomes partly differ. Central to the antimicrobial activity of neutrophils is the oxidative burst that generates reactive oxygen species (ROS) through the NADPH-oxidase multiunit enzyme complex (137). The dormant NADPH-oxidase consists of separate components distributed in the cytosol and the membranes of secretory vesicles. After phagocytosis, secretory vesicles that merge with the phagosome deliver cytochrome b, to which cytosolic proteins translocate to form a functional electron-transfer system, which catalyzes the reduction of molecular oxygen to superoxide ions. Azurophilic granula load MPO in the phagosome, with which the formed radicals react further to produce toxic molecules such as hypocloric acid (112).

NETs

Microbial killing was previously thought to be achieved exclusively trough uptake (phagocytosis) and activation of intracellular killing system (138). Recent findings suggest, however, that neutrophils also are able to form neutrophil extracellular traps (NETs) that bind, disarm and kill microbial pathogens without phagocytosis (139). NETs are composed of chromatin decorated with granular proteins. The formation of NETs is an active process dependent on the generation of ROS by the NADPH-oxidase.

Streptococcus pneumoniae – a pathogenic bacterium with many virulence factors

Streptococcus pneumoniae, or pneumococci, are common coloniser of the normal upper respiratory tract flora in humans, and most infants have been colonised with pneumococci before two years of age (140). However, under the right conditions pneumococci may spread and cause life-threatening disease. *S. pneumoniae* is among the most virulent bacteria in the human host and a common cause of diseases such as *otitis media*, pneumonia, meningitis and septicaemia. Despite treatment with antibiotics, invasive pneumococcal diseases have high mortality.

Classification of *S. pneumoniae*

Streptococcus pneumoniae is a Gram-positive, alpha-hemolytic facultative anaerobe, belonging to the *mitis* group of streptococci. Population genetic analysis have revealed that *S. pneumoniae* is one of several hundreds of evolutionary lineages forming a cluster separate from the other members of the *mitis* group *S. oralis* and *S. infantis* (141-143) (Fig. 5). The other lineages of this cluster were previously collectively referred to as *S. mitis* (Fig. 4), although it would be more appropriate to define them as separate species (143). While *S. pneumoniae* is among the most frequent microbial killers worldwide, the other species of the *mitis* group (*S. mitis*, *S. oralis* and *S. infantis*) are considered as apathogenic commensals of the oral cavity and/or upper respiratory tract.

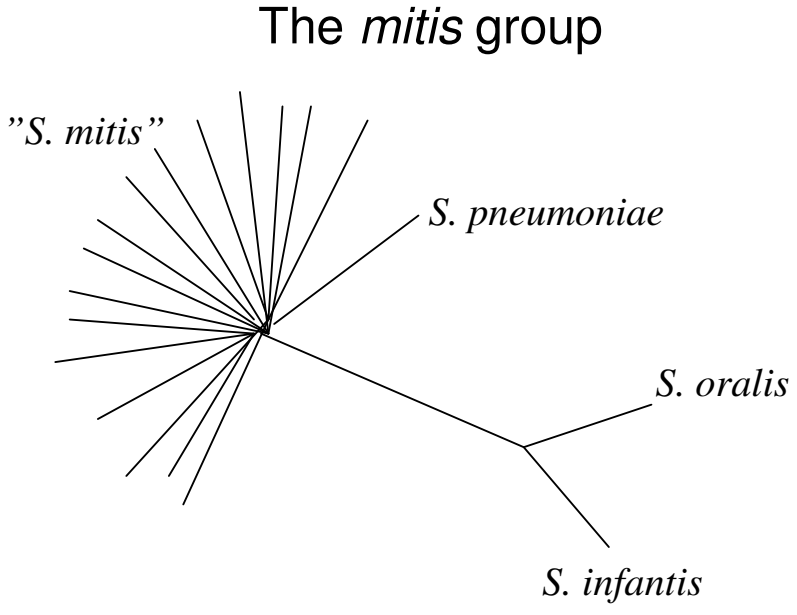

Figure 5. Phylogenetic tree showing Streptococcus pneumoniae and its close commensal relatives.

Diseases caused by *S. pneumoniae*

Otitis media

Otitis media is caused by bacteria ascending into the middle ear from the pharynx. Almost 95% of children have experienced at least one attack of acute otitis media by 3 years of age, and around half of these cases are caused by *S. pneumoniae* (144). Fluid accumulates in the middle ear space and inflammation occurs in the surrounding mucosa. Most cases resolve in 3-4 weeks, but occasionally complications such as meastoiditis (invasion of the skull bone) and sepsis occur (145). The serous that accumulates during otitis media may have different appearance and is classified as serous, purulent or mucoid. In acute *otitis media*, the fluid commonly contains high numbers of inflammatory cells (purulent fluid), while in chronic *otitis media* the fluid contains less inflammatory cells, but more mucous (145).

Injection of pneumococcal cell wall components into the middle ear of chinchilla has been shown to result in recruitment of inflammatory cells, lysozyme accumulation and epithelial metaplasia (146, 147). Toxic oxygen species released by activated neutrophils in the middle ear have been implicated in the epithelial metaplasia in *otitis media* (148).

Pneumonia

Pneumonia is a major cause of death worldwide, and *S. pneumoniae* infection is believed to be responsible for a majority of these cases (149).

Pneumococcal pneumonia occurs after spread of the bacteria from the nasopharynx to the lungs. Various pneumococcal virulence factors facilitate this process, including the adhesins psaA and CbpA (145). Alveolar macrophages confer the first line of defence, and are particularly important for asymptomatic clearance of small numbers of bacteria, as shown in a murine model of subclinical infection (150). Macrophage apoptosis is a prominent feature of pneumococcal infection and occurs via a nitric oxide (NO) dependent mechanism (151). If alveolar macrophages do not succeed in clearing the infection, an inflammation occurs. Upon interaction with bacteria, the macrophages produce mediators that attract large numbers of neutrophils to the lungs. Pneumococci readily gain access to the blood stream from the richly vascularised lung. Most deaths occurs within 5 days of illness (152), but splenectomised individuals may succumb within 18-24 h without symptoms (153). Of note, death may occur even after antibiotic treatment has begun, and no bacteria can be cultured from the lungs or blood. Patients that do recover usually have no permanent lung damage, despite the massive inflammatory response they have endured (154). Resolution is believed to occur after the appearance of anti-capsular antibodies which facilitate efficient phagocytosis. Neutrophils probably undergo apoptosis before being removed by

macrophages(155). The macrophages in turn are cleared by mucociliary transport, while fibroblasts migrate to repair the damaged lung interstitium.

Children, elderly and HIV-infected patients are more susceptible to pneumococcal pneumonia, but the disease can strike anyone. Other predisposing factors include neutropenia, hyposplenia, hypogammaglobulinaemia, complement and antibody deficiencies.

Meningitis

S. pneumoniae is the second most common cause of bacterial meningitis, and has the highest mortality rates (156). Furthermore, survivors are often left with neurological defects, such as hearing loss. Inflammatory mediators produced by recruited immune cells, such as ROS and NO species and inflammatory cytokines, are likely to be involved in causing the neuronal damage, and pneumolysin is directly toxic to neurons (157-160). Apoptosis is the chief mechanism of neural loss in pneumococcal meningitis (160).

Septicaemia

S. pneumoniae may reach the bloodstream via entry through the lung tissue or middle ear. Septicaemia is a serious, often mortal, complication of pneumococcal diseases.

Pneumococcal virulence factors

The tendency of *S. pneumoniae* to causes diseases is due to a pluritude of virulence factors, which facilitate adhesion, invasion and survival of attacks from the host immune system (Fig. 6).

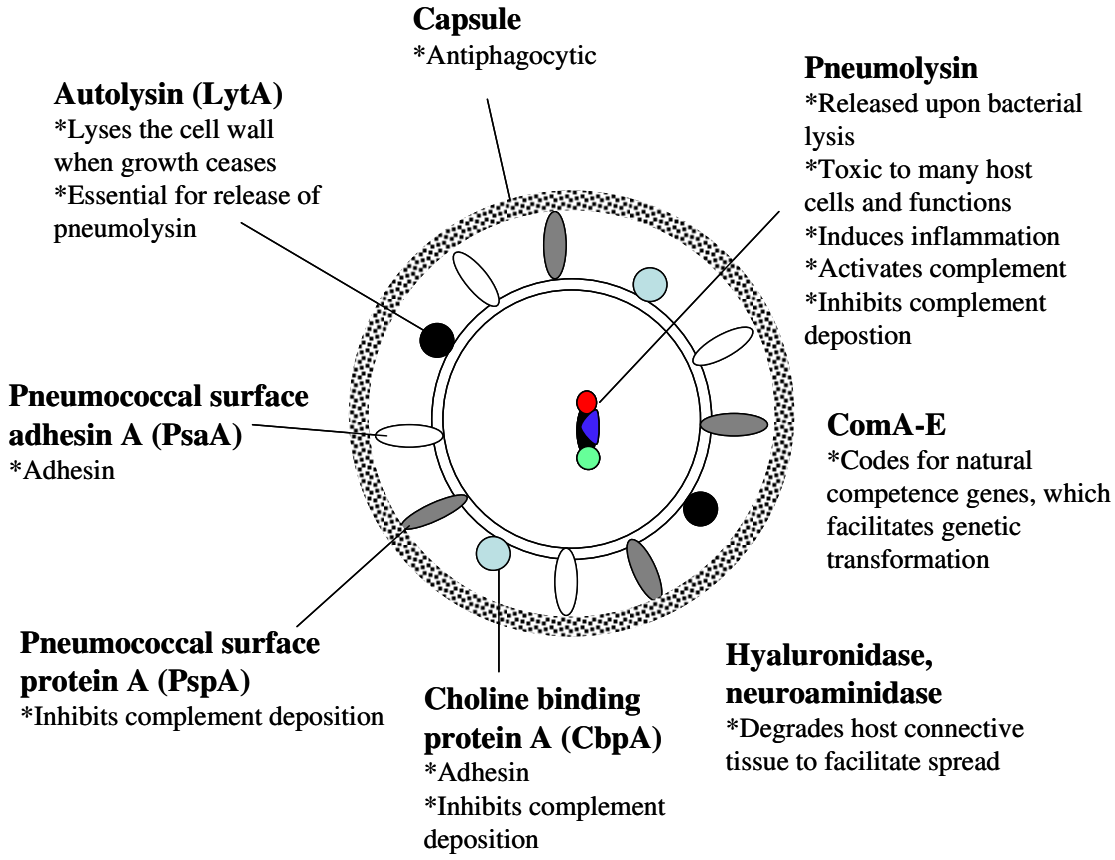

Figure 6. Important virulence factors of S. pneumoniae

Competence

S. pneumoniae has the ability to naturally become competent, i.e. able to pick up genes from the environment. The initiation of competence is regulated by the quorum sensing system, referred to as the ComABCDE (161, 162). This feature can explain the presence of the wide spectrum of virulence factors in pneumococci, and also the emerging increase in antibiotic resistance among *S. pneumoniae* (163). Hence, instead of just mutating existing genes, *S. pneumoniae* can take up new genes from the environment and adjust them for its specific needs.

Competence is a feature shared between *S. pneumoniae* and the other *S. mitis* strains, and is probably the reason for the existence of so many separate *S. mitis* lineages and the existence of strains that appear to be mixtures of *S. pneumoniae* and *S. mitis* (143). These strains, called *S. pseudopneumoniae*, resemble *S. mitis* in their house-keeping genes, but express various virulence factors normally restricted to *S. pneumoniae*, and are therefore more virulent than other *S. mitis* strains (143, 164).

The capsule

The thick carbohydrate capsule, which impedes phagocytosis, is the most important virulence factor for *S. pneumoniae*. Though pneumococci exist in encapsulated and non-capsulated forms, strains causing disease are almost exclusively encapsulated (165). The other members of the *mitis* group are not encapsulated.

There are over 90 known capsular serotypes, but 80% of invasive infections are caused by 8–10 serotypes (166). The capsule is hydrophilic and reduces interactions between bacteria and phagocytes; e.g. preventing iC3b that is deposited on the bacterial surface from interacting with complement receptors on phagocytic cells (153).

Antibodies against *S. pneumoniae* capsular polysaccharides are crucially important in host-defence, since they enable complement-dependent phagocytosis by activating the classical complement pathway. Patients with hypo-gammaglobulinaemia and other congenital deficiencies in immunoglobulin or complement factors have increased susceptibility to pneumococcal infections (167) (168) .

Pneumolysin

Pneumolysin is the second most important determinant of *S. pneumoniae* virulence. Pneumolysin is present in basically all clinical isolates of *S. pneumoniae*. Pneumolysin is a cholesterol-dependent cytolysin, and similar proteins can be found in a wide range of Gram-positive organisms, including strains of the Mitis group (169). A common feature of the cholesterol-dependent cytolysins is their ability to create large pores in cholesterol containing membranes, i.e. mammalian, but not prokaryotic, membranes. This interaction leads to insertion into the lipid bilayer, oligomerization and formation of transmembrane pores resulting in cell lysis (170). The *S. pneumoniae* cytolysin, pneumolysin, has evolved several unique characteristics. It is the only member of the cholesterol-dependent cytolysins that lacks a N-terminal secretion signal sequence. This makes release of pneumolysin to the environment dependent on lysis of the bacteria (171). However, a recent paper suggests that pneumolysin is localised within the cell wall of viable *S. pneumoniae* via hydrophobic interactions (172), and could be available for interactions with the host also before cell lysis. Pneumolysin has also been related to induction of

cytokine production via signal through TLR4 (55) and both to activate complement (173) and to inhibit complement deposition on the bacterial surface (174).

Mechanism of action	Consequence
Pore formation – lytic concentration	*Cell cytotoxisity, cell type and concentration dependent
Pore formation – sublytic concentration	*Inhibition of ciliary beating *Apoptosis of neurons
TLR4 stimulation	*Induction of proinflammatory cytokines
Complement activation	*Activates complement after binding the Fc-part of immunoglobulins and Cq1

Table 2. Examples of effects of pneumolysin on the host.

Injection of purified pneumolysin to laboratory animals mimics many of the characteristics of pneumococcal diseases (175, 176). Further, though not as fully as capsular antibodies, antibodies to pneumolysin protects from pneumococcal disease (177). Pneumolysin may exert a number of actions on its host depending on its concentration in body fluids (Table 2). At high concentrations (1-10 µg/ml) the toxin shows lytic activity, and is toxic to virtually all cells in the body. At low concentrations (1-100 ng/ml) pneumolysin is capable of a range of immunomodulating activities including inhibition of antibody production (171, 178), induction of cytokine production (179), complement activation (173), induction of apoptosis in neurons (160). Some of these activities depend on its pore-forming (haemolytic) ability, while others are independent of this (Table 2).

In mouse models of pneumococcal disease, mutant strains lacking pneumolysin are strikingly less virulent than wild type strains (176, 180). *S. pneumoniae* expressing pneumolysin with mutations affecting cytolytic and/or complement activating properties also have reduced virulence (181-183). Certain clinical isolates of *S. pneumoniae* have been found to bear mutations in their pneumolysin gene, which reduces the haemolytic activity of pneumolysin (184-187). Injection of haemolytic mutant pneumolysin to the lungs of laboratory animals causes less cell damage and cytotoxicity of epithelial cells, than did native pneumolysin (188), but pneumococci expressing pneumolysins lacking haemolytic or complement binding activities are still more virulent than strains lacking pneumolysin (181). Thus, multiple haemolytic-dependent and independent actions of pneumolysin appear to be important for its virulence.

CBPs, including autolysins

The cell wall teichoic acids (TA) and lipoteichoic acids (LTA) are substituted with choline moieties in *S. pneumoniae* and the other members of the *mitis* group, as well as several other Gram-positive bacteria colonising the respiratory tract (189). Phophoryl choline functions as an adhesion molecule, but also as an anchor for non-covalent attachment of choline-binding proteins (CBP). In *S. pneumoniae*, there are at least 10 different types of CBPs, out of which several are important in virulence. These include CbpA and PspA, which are important for adhesion and inhibition of complement deposition (145, 189). These virulence associated CBPs are unique for *S. pneumonia.*

Another group of CBPs, collectively referred to as autolysins, are enzymes that degrade cell wall peptidoglycan. They include LytA, LytB, LytC and LytD (CbpD), which have different roles in the life cycle of the bacterium. Many of the autolysins are shared between *S. pneumoniae* and *S. mitis* (189).

LytA is called the major pneumococcal autolysin and is expressed by the vast majority of *S. pneumoniae* isolates. LytA is a N-acetyl muramic acid L-alanine amidase that hydrolyses amide bonds in the peptides that connect the peptidoglycan strands in the cell wall (190). It is normally absent in *S. mitis* (143), although certain *S. mitis* isolates express a variant of the LytA gene that differs from *S. pneumoniae* LytA in its requirements for activation (189, 191). The pneumococcal LytA gives the bacterium an odd characteristic. When bacterial growth ceases, their cell wall bound LytA spontaneously becomes activated and lyses the bacterial cell wall. This also occurs in the presence of β-lactam antibiotics, which is the reason for the high sensitivity of most pneumococci to penicillin (192).

The precise mechanism leading to spontaneous activation of LytA in the stationary phase of growth is not clear. LytA is synthesised in a low-activity E-form, but when present in the cell wall it appears to be exclusively in the active C-form (189). Since lysis occurs only during the stationary phase, the activity of LytA must be inhibited during the exponential phase of growth. Even externally added LytA can not lyse bacteria during exponential growth, though it readily lyses bacteria in stationary phase (193). A hypothesis is that LytA bound to TA hydrolyses the cell wall, while LytA bound to LTA inhibits this activity. When bacterial growth ceases, lipid anchored structures, such as LTA, are shed which would result in activation of the TA bound LytA.

It may seem odd that a bacterium would benefit from spontaneous lysis. However, the LytA-mediated lysis occurs late in the bacterial life cycle, and the presence of dead lysed bacteria, rather than dead intact bacteria, may be beneficial for survival of relatives. Indeed, LytA negative mutants of *S. pneumoniae* are less virulent than wild-type strains in several mouse models of *S. pneumoniae* infection (194-197).

Two hypothesis have been put forward; that the cytoplasmic component pneumolysin is released leading to toxic functions (171, 198) or that cell wall degradation products such as peptidoglycan-fragments and lipotechoic acid (LTA) increase the inflammatory response (199-201).

The other pneumococcal autolysins include LytB, LytC and LytD. They are often also found in S. *mitis* strains. LytB is involved in daughter cell separation (202). LytD is expressed only when bacteria become competent, and functions by activating LytA and LytC on neighbouring bacteria making genes available for recombination (203). As *S. mitis* has the ability to become naturally competent, but does not spontaneously lyse during stationary cell growth, it seems likely that the *S. mitis* autolysins participate in the process of gene-transfer, and that the spontaneous lysis of pneumococci has evolved from this function.

Genetic variation

It has been suggested that virulence factors may vary in importance for pneumococci of different capsule types and genetic backgrounds (204). Furthermore, *S. pneumoniae* can undergo spontaneous phase variation between an opaque and transparent phenotype, with different virulence factor expression (205). The transparent phenotype may be adapted for colonisation, whereas the opaque phenotype may be better for invasion and spread (145).

Host defence to *S. pneumoniae*

Antibodies

The encapsulated *S. pneumoniae* is regarded as a classical extracellular pathogen. Elimination of pneumococci depends on opsonised phagocytosis and killing by phagocytes, i.e. alveolar and tissue macrophages and recruited neutrophils. Antibodies against capsular structures of *S. pneumoniae* are crucially important for resolution of the infection, since they facilitate efficient phagocytosis. Antibodies that bind to the surface of pneumococci, initiate complement-dependent opsonisation and activation of the classical complement pathway. The crucial importance of antibodies in host defence is easy to understand, since most, if not all, types of B cell defects increases the risk for pneumococcal diseases (167, 168, 206). Further, deficiencies in the early components of the classical pathway of complement and C3 deficiency predispose for *S. pneumoniae* infections (206).

Phagocyte activating cytokines

S. pneumoniae is not sensitive to antibody-mediated lysis, but opsonised bacteria must be taken up and killed by phagocytes. Animal studies have shown that mice deficient in IL-12, TNF or IFN-γ, cytokines important for enhancing the bactericidal mechanisms in macrophages, are more susceptible to pneumococcal infections (123-126). In human, a patient with IL-12 deficiency was shown to

suffer from recurrent episodes of pneumococcal diseases (207). Further, neutralisation of TNF has been shown to increase the bacterial burden and mortality in mice infected with *S. pneumoniae* (208-210). Similarly, neutralisation of TNF in clinical trials during *S. pneumoniae* sepsis had detrimental effects (211).

Apart from activating phagocytes, TNF is important in recruiting neutrophils and IL-12, TNF and IFN-γ may play a role in humoral immune responses to *S. pneumoniae*. Hence, mice lacking functional IL-12, TNF or IFN-γ showed substantially reduced production of antibodies during pneumococcal infection (212) and addition of IL-12 to intranasal pneumococcal polysaccharide conjugate vaccines increased both systemic and mucosal immune responses and conferred enhanced protection against invasive infection (213).

In accordance with the important role for cytokines in the pneumococcal defence, IRAK-4 mutations are associated with increased risk for pneumococcal diseases. Signalling via the MyD88-dependent pathway is abolished in these patients, leading to impaired cytokine production to most TLR-ligands. In a study of 28 IRAK-4-/- patients, 80% suffered from invasive pneumococcal diseases, which often were recurrent, and half of the patients died from their infections. Apart from severe staphylococcal disease (30%), other infections agents were rare (214). Further, impaired NF-κB activation in patients is also associated with a high risk of pneumococcal diseases (206).

In mice, immunity to *S. pneumoniae* is impaired in the absence of TLR2 and TLR4 signalling (215-218). *S. pneumoniae* contains several ligands that may signal via TLR2 (lipoproteins, LTA, PG) and pneumolysin, which may signal via TLR4 (55). Signalling via TLR2 and TLR4 may be partly redundant, since double KO mice get more severe pneumococcal disease than single KOs (215-218). In both pneumococcal and meningitis models, KO mice show reduced cytokine levels and reduced pathology early in infection, but clearance of bacteria is impaired and the animals succumb to bacteraemia (216, 218). Also transgenic mice with impaired NF-κB activity are less able to control *S. pneumoniae* diseases (219).

Adaptive T cell responses

When innate immune reactions are not sufficient to rapidly eliminate infectious agents, help from the adaptive immune system is needed. The adaptive immune system consists of helper, cytotoxic and regulatory T cells, B cells and antibody-producing plasma cells, all of which can recognize and react to millions of different specific structures, termed antigens. The antigen-specificity of T and B cells lays in their T cell receptors (TCRs) and B cell receptors (BCRs), respectively. Although one lymphocyte expresses only a single receptor, the diversity of the population confers the capacity of lymphocytes to recognise almost infinite numbers of antigens. As only very few lymphocytes are reactive against each pathogen, those rare cells must upon activation be clonally expanded in order to exert efficient immune reactions. Thus, primary adaptive immune responses are not measurable until several days after antigen accounter. Following a second exposure, however, more antigen-specific clones are present and a more rapid and vigorous lymphocyte response is seen. This is the hallmark of an adaptive memory response. The pathogen is eliminated by recruitment of innate killing mechanisms by the adaptive response. Thus, complement is activated when antibodies bind antigen and phagocytes may be activated by antigen-specific T cells producing IFN-γ. In addition, virus infected cells may be lysed by cytotoxic T cells. Adaptive immune responses can also be induced to antigens of non-microbial origin, such as in food proteins and inhaled air. Ideally, immune reactions to innocuous antigens should be non-aggressive or tolerogenic, which often, but not always, is the case. To confer tolerogenic reactions to innocuous antigens is the task of regulatory T cells.

Activation of naive T cells

T cells are selected in the thymus

All types of white blood cells, including lymphocytes, are formed in the bone marrow. While B cells fully mature in the bone marrow, T cell progenitors migrate in an early stage to the thymus for further differentiation. In the thymus, T cells with a useful repertoire of T cell receptors (TCR) are selected based on two criteria; they should be able to bind to self MHC (positive selection), but should not bind with too high avidity to presented self-antigens (negative selection). T cells leaving the thymus after passing this selection process are called naïve, since they have never encountered their specific antigen.

Presentation to naïve T cells occurs in secondary lymphoid organs

Naïve T cells circulate in the body, until they encounter their cognate antigen presented by an antigen-presenting cell (APC). This first encounter normally takes

place within specialised lymphoid tissues, called secondary lymphoid organs, which are organised to facilitate interactions between lymphocytes and APCs. The secondary lymphoid organs include the spleen, lymph nodes and Peyer´s patches, which are lymphoid structures distributed along the intestines.

APCs that are scatted in the skin and mucosa take up and bring antigen to draining lymph nodes for presentation to T cells. In addition, resident APCs in the secondary lymphoid organs capture antigen for presentation within this compartment. Naïve T cells that circulate in the blood vessels enter into secondary lymphoid organs via high endothelial venules. Within the lymph nodes they scan the APCs in search of their specific antigen and if their antigen is not presented, they leave the lymph node via the efferent lymph and return to the blood. If a T cell does encounter its cognate antigen, it stays in the lymph node, becomes activated, and starts to proliferate. The daughter cells mature into effector cells that may enter into the follicle area to help B cells produce antibodies, or leave the lymph node and migrate to sites of, for example, inflammatory focus, where they perform their functions.

Activation of naïve T cells occurs through three signals

The first necessity for activation of a $CD4^+$ T cell is the recognition by the T-cell receptor (TCR) of antigenic peptides presented by major histocompatibility complex (MHC) class II molecules on an APC. However, this is not enough to efficiently activate naïve T cells and presentation of antigens in a non-stimulatory fashion may instead lead to anergy. Second signals, or co-stimulation, are needed to activate the T cells. Co-stimulation can be provided by the triggering of CD28 on the T cell by CD80 and CD86 expressed on the APC. In addition, third signals, such as cytokines and other soluble mediators or costimulatory molecules, are important to polarize, or directs T-cell differentiation, into various effector phenotypes. This third signal may be provided either by the APC presenting the antigen, or by other cells in the vicinity, including NK and NKT cells, γδ T cells and macrophages.

Both signal two and three, i.e. upregulation of co-stimulatory molecule expression and production of polarising cytokines, are likely to be enhanced by the presence of microbes and microbial products triggering innate immune cells through stimulation of receptors such as the TLRs. Different microbes may trigger different polarising signals. Gram-negative bacteria appear to induce stronger up-regulation of costimulatory molecules on DCs compared to Gram-positive bacteria (220, 221). This may be related to their ability to, via LPS stimulation of TLR4, trigger both the MyD88-dependent and TRIF-dependent activating pathways in the APC (222).

Though monocytes produce higher levels of IL-12 and TNF in response to Gram-positive, than to Gram-negative bacteria (103, 128), the pattern is less clear upon bacterial stimulation of DCs. Compared to monocytes, in vitro differentiated

moDCs produce higher levels of IL-12 and TNF in response to Gram-negative bacteria (59), while similar or to some strains lower levels of IL-12 and TNF are produced in moDC, compared to monocytes, in response to Gram-positive bacteria (59, 220, 221). Whether or not Gram-positive and Gram-negative bacteria induce different types of T cell activation is not clear.

Antigen presenting cells

Antigens are presented to T cells by antigen-presenting cells (APCs) on MHC-molecules. There are two types of MHC-molecules, the MHC I and MHC II. $CD8^+$ T cells recognise peptides in the context of MHC I, while $CD4^+$ T cells recognise peptides exposed on MHC II.

In general, peptides derived from endogenous proteins and infecting viruses are loaded on MHC I, but also exogenous antigens can be directed to MHC I via a process referred to as cross-presentation. While all cells in our body express MHC I and present endogenous antigens on this molecule, only APCs have the capacity to cross-present antigens.

MHC II is only expressed by antigen-presenting cells. Antigens that are taken up by APCs are degraded in phagosomes. Vesicles from the endoplamatic reticulum, containing MHC II, merge with the phagosome and antigen peptides are loaded on the MHC, which is transported to the surface where it is exposed to $CD4^+$ T cells.

Dendritic cells

Dendritic cells (DCs), which in mice are characterised by expression of the α-chain of complement receptor 4, CD11c, are efficient stimulators of naïve T cells (223-225). They were originally identified in secondary lymphoid organs by Steinman *et al;* as cells with long dendrites, high MHC II expression and pronounced capacity to stimulate T cells (226, 227).

The conventional DCs, defined as $CD11c^+B220^-$ cells, can be divided into lymphoid-tissue resident DCs and migratory DCs. The lymphoid-tissue resident DCs appear to have developed directly from bone marrow progenitors, while migratory DCs can mature either directly from bone marrow progenitors, or via monocytes arriving to the tissue from the blood stream (106, 107). While the lymphoid-tissue resident DCs are thought to both capture and present antigens within secondary lymphoid organs, the migratory DCs are scattered in tissues, where they capture the antigens before migrating to a lymph node to present it to T cells (228). Thus, most secondary lymphoid tissues contain a mixture of lymphoid-

tissue resident DCs and migratory DCs. However, as the spleen is not drained by the lymphatics, it is considered to contain only tissue resident DCs (Fig. 7).

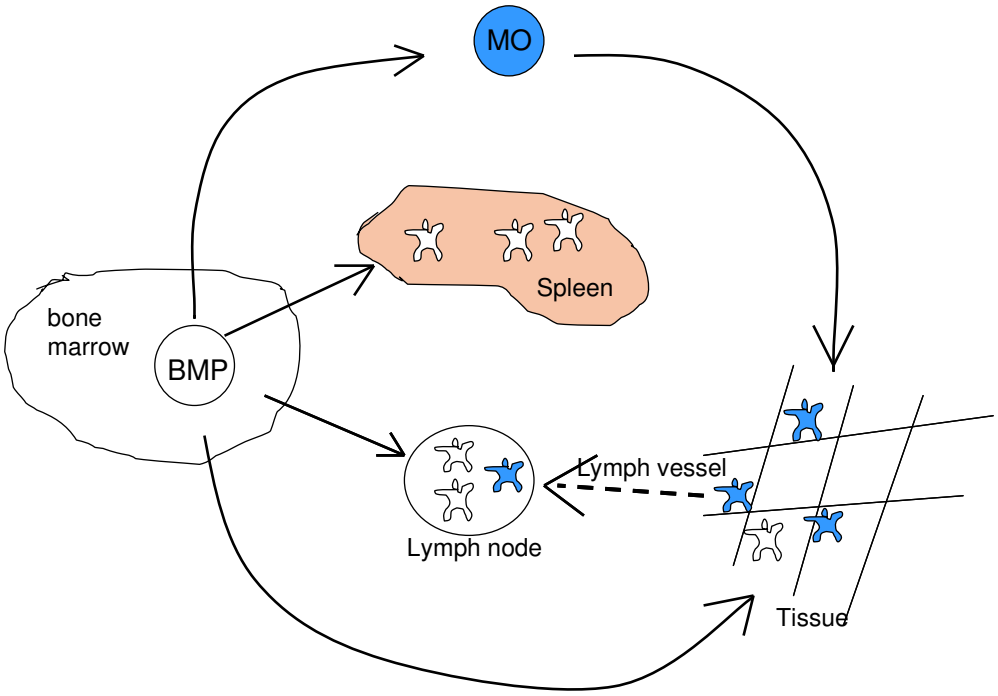

Figure 7. The lymphoid-tissue resident DCs in the secondary lymphoid organs develop directly from bone marrow precursors (BMP), while migratory DCs in tissues can develop also from monocytes (MO). Migratory DCs wander to draining lymph nodes, but not the spleen, to present captured antigens.

The resident DCs as well as the migratory DCs can be further subdivided into several subsets. Resident DCs include $CD8\alpha^+$ and $CD8\alpha^-$ DCs, where the $CD8\alpha^+$ subset more readily produces IL-12 and is connected with a higher grade of Th1 polarisation than the $CD8\alpha^-$ DCs subset, which instead promote more Th2 polarisation (229-231). The phenotype of migratory DCs are different in the skin, *lamina propria* and lungs, but they may all be divided into $CD103^+$ and $CD103^-$ subset.

Dendritic cells, both in peripheral organs and in secondary lymphoid organs, have high phagocytic and endocytic activity, though the phagocytic activity may be down-regulated upon DC maturation (232). Selective expression of phagocytic receptors on different DC subtypes may result in selective uptake of different types of antigens and particles. For example $CD8\alpha^+$ DCs have been shown to be more efficient than $CD8\alpha^-$ DCs in taking up apoptotic bodies, while the opposite has been demonstrated for phagocytosis of *Leishmania* parasites (233, 234). The main

function of phagosomes formed in DCs is not to kill the prey, but also to process it and generate peptides that can be presented on MHC I and MHC II to T cells. In general, DC phagosomes are less proteolytic than macrophage phagosomes, maintaining peptides to be presented on the MHCs (112). The pH of the DC phagosome is higher than in that of the macrophage, and a high pH appears to be important particularly for cross-presentation of MHC I (235). The $CD8\alpha^+$ DC are better than the $CD8\alpha^-$ DC in cross-presenting soluble antigens and apoptotic cells to $CD8^+$ T cells, possibly due to the higher pH of their phagosomes. The $CD8\alpha^-$ DC preferably activate $CD4^+$ T cells via presentation on MHC II (233, 236, 237). Similarly the migratory subsets handle antigen differently, with the $CD103^+$ subset being the most efficient cross-presenters of exogenous antigen to $CD8^+$ T cells (106, 228, 229, 238-241).

Plasmacytoid dendritic cells

A non-conventional DC type is the plasmacytoid DC, which has a plasmacytoid morphology, is $CD11c^{low}B220^+Ly6C^+$ and produces IFN-α in response to virus stimulation. Plasmacytoid DC appears to be of importance in virus defence. They efficiently present endogenous antigens including those derived from viruses infecting the pDCs to $CD4^+$ and $CD8^+$ T cells, but appear to be less efficient than cDCs in capturing and presenting exogenous antigens (242, 243).

Monocytes

The monocyte populations in the mouse can be divided into $Ly6C^{hi}$ and $Ly6C^{lo}$ monocytes, both of which can develop into macrophages and DCs in the tissue. Due to different chemokine receptor expression, their migration patterns differ. While the $Ly6C^{lo}$ monocytes continuously migrate to tissues during steady state, the $Ly6C^{hi}$ monocytes are recruited in high numbers during many inflammatory conditions. They leave the blood stream and enter the inflamed tissue and secondary lymphoid organs (244).

For example, alum injection to the peritoneum results in accumulation of $Ly6C^{hi}$ monocytes. When alum was injected together with an antigen, the recruited monocytes capture the antigen, migrate to the draining lymph nodes, upregulate their expression of MHC II, costimulatory molecules and CD11c and become efficient stimulators of $CD4^+$ T cell (25, 245). The potent T cell activation in the absence of TLR ligand stimulation is probably the reason for the Th2 polarising effect of alum.

Monocytes accumulating in secondary lymphoid organs during infections have been shown to produce TNF and iNOS and participate in phagocytosis and killing of bacteria, but not generally in priming T cells (246-248).

B cells and NK cells

B cells express MHC II and can present antigens to $CD4^+$ T cells. The high-affinity BCR enables the capture specific antigens at low concentrations and signalling through the BCR also activates efficient antigen processing in the B cells (249-251). In addition, B cells may pinocytose and present non-specific exogenous proteins to T cells, but this mechanism is by far less efficient than the receptor mediated presentation (252-254). However, special B cell subtypes might be better equipped for antigen-presentation than other (26). Also certain types of activated NK cells have been suggested to have the ability to activate T cells (24, 255).

Macrophages

Macrophages generally express low levels of MHC II. Though they were the first APC identified (256), they are compared to dendritic cells regards as poor presenters of antigens (227). The poor ability of macrophages to induce T cell activation may relate to their extremely high proteolytic capacity. In macrophages, the pH drops to values around 5.5 within the first 30 min of engulfment, and most proteins are rapidly degraded into amino acids.

Macrophages are derived from monocytes, and share many surface markers, such as CD11b, with neutrophils and dendritic cells. Though different researches use different criteria to define macrophages, most agree that macrophages express high levels of F4/80 and CD68 and are highly autoflourescent (257, 258).

Inhibitory functions of macrophages

Apart from there role in activating immune responses, monocytes and macrophages are also involved in terminating or inhibiting responses. In some occasions, such as during cancer, this is detrimental and limits the effectiveness of immunotherapy. Myeloid-origin cells, termed myeloid-derived suppressor cells (MDSC), that inhibit both adaptive and innate immunity have been shown to accumulate in the blood, lymph nodes, bone marrow and at tumour sites in most patients and experimental animals with cancer (259). Also terminally differentiated macrophages in the lungs and peritoneal cavity have been shown to inhibit lymphocyte activation, but at these locations the inhibitory effect may play a physiological role.

Alveolar and peritoneal macrophages

The alveolar macrophages have long been recognised, not only for their phagocytic capacity, but also for their ability to down-regulate local immune reactions in the lungs (260). This appears to be an important homeostatic mechanism, as *in vivo* depletion of alveolar macrophages is associated with dysregulated local immune reactivity and hyper-responsiveness to inhaled aerosols (261). Upon incubation with allergen or mitogen stimulated splenocytes *in vitro*, the alveolar macrophages

dose-dependently inhibit lymphocyte proliferation. The inhibition is reversible and in rodents mainly dependent on NO secretion by the macrophages (262). Also human alveolar macrophages have been shown to reversibly inhibit PBMC proliferation in response to mitogen and allergen. However, in this case the inhibition was not dependent on NO (263).

Similarly, peritoneal macrophages have also been described to inhibit lymphocyte proliferation (262, 264, 265), but less efficiently than alveolar macrophages (262). This was suggested to depend on NO secretion and catabolism of the essential amino acid tryptophan (265), or prostaglandin secretion (264). Furthermore, both resident macrophages in the peritoneal cavity and in the lungs constitutively produce low levels of TGF-β (266-268), which can be increased by stimulation with IL-2 (268). They can also secrete IL-10 when stimulated (265).

T cell subsets

Conventional T lymphocytes

Conventional T cells have the αβ chain T cell receptor. Each chain is produced after somatic recombination of variable (V) and joining (J) gene segments. A conventional T cell becomes activated after meeting its cognate antigen on a MHC molecule, presented in a stimulatory fashion by an antigen-presenting cell (APC). The MHC molecules of class I and II present short peptides derived from processed cytosolic (class I) or endosomal (class I and II) antigens. T cells also express either CD4 or CD8 on their surface. CD4 stabilises interactions with MHC II, while CD8 does the same for MHC I, with makes the $CD4^+$ T cells MHC II restricted, while the $CD8^+$ T cells are MHC I restricted. Upon receptor stimulation, the TCR conveys down-stream signals via the receptor associated CD3 molecule, leading to activation and proliferation of the T cell.

Activated $CD4^+$ T cells exert their actions mainly by secretion of cytokines. These cytokines regulate, or "help", responses of other immune cells, so the $CD4^+$ T cells are therefore also called helper T cells. Depending on various factors the activated $CD4^+$ T cells can be polarised to become Th1, Th2, Th17 or regulatory T cells, which secret different patterns of cytokines and have distinct effects on immune response.

$CD8^+$ T cells become activate by peptides presented on MHC I by an APC, but almost all cells in our body express MHC I and expose cytosolic antigens on this receptor. Activated $CD8^+$ T cells can therefore detect and kill for example virus infected cells exposing the virus on their MHC I. $CD8^+$ T cells also produce cytokines when activated, e.g. IL-2 and IFN-γ.

Non-conventional T lymphocytes

In addition to the conventional T cell subsets activated by peptides presented on MHC I and II, there are also subsets of non-conventional T cells that recognise other types of antigens and by other means. The subsets of non-conventional T cell include γδ T cells and NKT cells.

γδ T cells

Instead of expressing the αβ TCR, some T cells have a γδ TCR. They are mainly localised in the gut and skin epithelium. The γδ TCR detects heat-shock-proteins, small bacterial phosphoantigens and certain peptides and lipids, not always in the context of an antigen presenting cell (269, 270). The γδ T cells elicit rapid cytokine responses, as well as potent cytotoxic activity upon activation (271, 272).

NKT cells

NKT cells are a diverse group of αβ TCR cells. Their name signifies their combined T cell and NK cell related properties. The most common "classical" NKT cells express an invariant α chain together with a limited number of β chains. They recognise lipid-antigens, the most well known being α-GalCer, presented on CD1d (273), a MHC-like molecule which exists in five isotopes in human (CD1a-e), but only one in mice (CD1d) (274). Other NKT cells have a more variable receptor, but not as diverse as conventional T cells.

Only a few bacterial ligands able to engage the TCR have been identified, but NKT cells are activated by various infections agents and usually contribute to clearance of the infectious agent (273). Activated NKT cells produce cytokines and kill cells, without the need for extensive cell division or differentiation, unlike conventional lymphocytes. In addition to the CD1-mediated activation, NKT cells may also be activated by cytokines, such as IL-12 and IL-18, even in the absence of DCs (275). As NKT cells can produce cytokines such as IFN-γ, IL-4 and IL-17, they may be important both in activating DCs and for determining the polarisation of activated $CD4^+$ T cell (271, 272).

$CD4^+$ T cell polarisation

A conventional $CD4^+$ T cells that meets its cognate antigen presented in the context of MHC II starts to proliferate. Based on the nature of the antigen and the APC and the conditions prevailing in the tissue during presentation, the T cell becomes polarised, meaning that a certain cytokine profile and reaction pattern becomes imprinted in the cell due to expression of a certain transcription factor. For many years, only the Th1 and Th2 polarisation was known, but during recent years a large number of additional T cell polarisations have been characterised (Fig. 8).

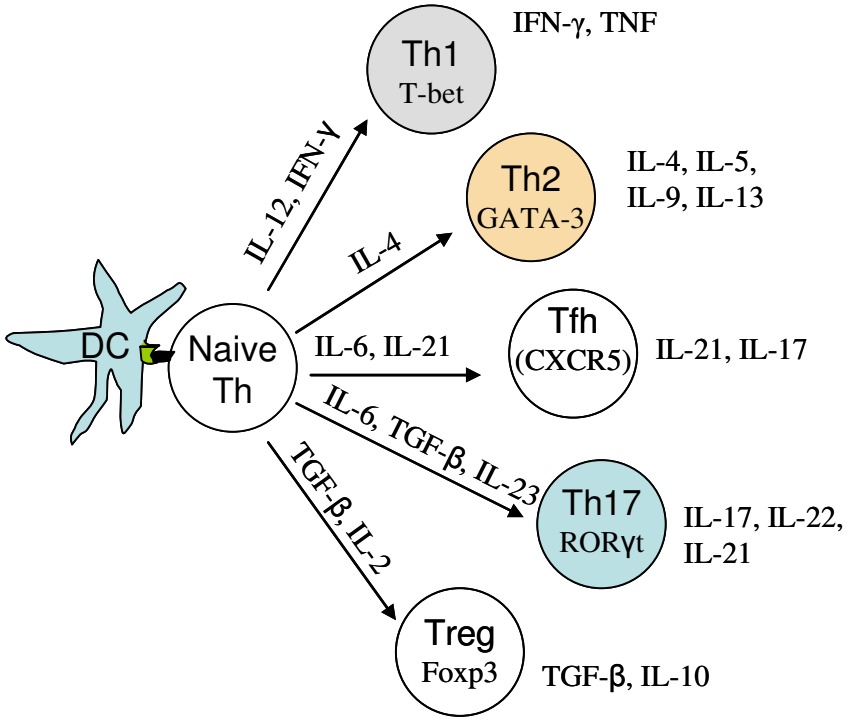

Figure 8. When a naïve CD4$^+$ T cell meets its cognate antigen presented by a DC, it can become polarised into one of several different effector T cells.

Th1

Th1 cells express the transcription factor T-bet and produce mainly IFN-γ, but also TNF. IL-12, which can be produced by macrophages and dendritic cells, is the major factor driving Th1 polarisation, but IFN-γ, IL-18, IL-27 and IFN-α also play a role. Effector Th1 cells activate macrophages and neutrophils to become more efficient killers. In addition, Th1 cells stimulate NK cells, CD8$^+$ T cells and promote B cells to produce opsonising subclasses of IgG antibodies. Though Th1 cells are important to eradicate intracellular pathogens and extracellular Gram-positive bacteria, Th1 responses have also been associated with autoimmune diseases, as well as contact dermatitis and chronic atopic eczema (276).

Th2

Th2 cells express the transcription factor GATA-3 and produce the cytokines IL-4, IL-5, IL-9 and IL-13. The IL-4 required for polarisation of Th2 cells can be produced by mast cells, basophiles, eosinophils, activated NKT cells and differentiated Th2 cells, but the source of IL-4 in the lymph node during initial activation of naïve CD4$^+$ T cells in unclear. Effector Th2 responses are involved in the defence against certain helminths and other extracellular parasites, but a predominant Th2 response is also linked to atopic allergic reactions, as it produces IL-4 that stimulates B cells to produce IgE antibodies and IL-13 which enhances mucus production (276).

Th17

The recently described Th17 cells express the transcription factor RORγt. In mice, Th17 polarisation is induced by TGF-β in combination with IL-6, while IL-23 may be involved in expanding the population. In human IL-1β, IL-6 and IL-23 appear to be the driving forces for Th17 differentiation (276). Recently, also PGE_2 was identified as a mediator involved in Th17 differentiation (277). Effector Th17 cells produce IL-17 and IL-22. The major effect of IL-17 is the induced release of CXC chemokines, including IL-8, from fibroblasts and endothelial cells. The release of these chemokines is augmented by TNF and results in recruitment of neutrophils (278). IL-22 induces release of anti-microbial peptides from epithelial cells improving host defences (278). Th17 cells are involved in the defences of the lungs and intestines to Gram-negative bacteria and fungi, but are increasingly recognised as participating in autoimmune diseases (278).

Tfh

A T cell lineage specialised for helping B cells to mature, has recently been described and termed follicular helper T cells (Tfh). IL-6 and IL21 appear to be important for their differentiation. The Tfh express CXCR5 and therefore migrate to the lymph node follicles to support antigen-specific B cell maturation. Tfh interact with B cells that present their cognate antigen on MHC II and signals via receptors such as OX40, ICOS and CD40L to induce B cell maturation. Tfh have been shown to secrete IL-17 and IL-21, but are not though to be a major players in directing the immunoglobulin isotype switch. Instead factors secreted by cells in the vicinity, including Th1, Th2 and Treg, influence the isotype switch. For example, in mice IL-10 and TGF-β are important for switch to IgA, IL-4 for switch to IgE and IgG1, and IFN-γ for switch to opsonising isotypes such as IgG2a (279).

Treg

To prevent excessive or unwanted immune reactions, the adaptive immune system needs to be regulated. During T cell development a subset of $CD4^+$ T cells, called regulatory T cells (Tregs), with the ability to down-regulate immune responses upon TCR engagement, is established. Tregs express the transcription factor Foxp3 and display high surface expression of CD25 and intracellular CTLA-4. Tregs are selected in the thymus based on fairly high avidity for self-antigens, which gives them the property to antigen-specifically hinder immune responses to self-antigens (280). Fatal autoimmune diseases, inflamed bowel, high IgE levels and eczematous skin lesions are associated with mutations of Foxp3 in human as well as and mice (281, 282).

How the response to innocuous food and environmental antigens is controlled is unclear. Self-specific thymus-derived Tregs may suppress effector T cells with other specificity, so called bystander tolerance. Further, naive non-self-reactive $CD4^+$ T cells can be converted to Foxp3 expressing induced Tregs (iTregs) in the

periphery (283, 284). TGF-β is a driving force for such iTreg induction and also expression of IDO by the APC may lead to generation of iTregs (280, 283-285). The suppressive effects of iTregs have been suggested to involve secretion of cytokines, such as IL-10, while natural Tregs mainly use contact dependent mechanisms.

Other types of T cell polarisations

T cells secreting only IL-22, termed Th22, have been identified in the skin. This polarisation is dependant on expression of the aryl hydrocarbon receptor transcription factor AhR (286, 287). Also T cells secreting IL-9, but not IL-4, IL-5 or IL-13, have been described and denoted Th9. The transcription factor involved in this phenotype has not been identified (288).

Furthermore, using intracellular staining, polarised T cells have been shown to be able to express cytokines proposed to belong to different T cell linages simultaneously. This is especially common for IL-10, which can to be expressed by T cells together with either IFN-γ, IL-17 or IL-4 (289-291). Polarised T cells are at least partly plastic. Established Th2 cells may under the influence of TGF-β may be diverted to Th9 cells (288) and Tregs can be converted into Th17 cells (292, 293), a process that appears to be inhibited in the presence of retinoic acid (294). Thus, the definition of distinct "subsets" of T cells with set cytokine profile is an oversimplification.

T cell responses at the mucosa

The mucosal immune system consists of non organised lymphoid tissue called lamina propria, as well as organised lymphoid structures such as the Peyer´s patches (PP) and mesenteric lymph nodes (MLN). It has long been recognised that orally ingested soluble protein antigens have low immunogenicity and instead tend to induce tolerance, i.e. oral tolerance (295-298). The induction of oral tolerance is dependent on MLN, but can occur in the absence of PP (299, 300).

The mucosal surfaces of the body are constantly exposed to a variety of microbial and food antigens. The need of protective immunity to the intestinal bacteria must be balanced by the necessity not to mount aggressive immune reactions to innocuous antigens, which could result in allergy. It is therefore not surprising that immune responses in the mucosa differ from the immune reactions in peripheral lymphoid organs. Broadly, the immune reactions in the intestinal mucosa are characterised by production of high levels of IgA against bacteria, and iTreg generation towards soluble protein antigens. In addition, effector cells producing IFN-γ and IL-17 are present in the lamina propria, but absent in germ free mice (301).

AIMS OF THE STUDY

The main objective of this thesis was to study and compare the immune responses elicited by Gram-positive and Gram-negative bacteria in general, and the pathogenic Gram-positive bacterium *Streptococcus pneumoniae*, in particular.

The specific aims were:

- To study the intracellular signalling pathways induced in monocytes by Gram-positive bacteria that result in IL-12 production.

- To study the role of the *Streptococcus pneumoniae* virulence factors autolysin and pneumonlysin in innate immune reactions, such as phagocytosis, induction of phagocyte activating cytokines, and oxygen radical production.

- To compare the $CD4^+$ T cell activation and polarisation induced by different APCs to an antigen present in Gram-positive or Gram-negative bacteria, or in its soluble form.

MATERIALS AND METHODS

Bacteria and bacterial components

Bifidobacteria (I)

In paper I, strains from two bifidobacterial species, *B. adolescensis* and *B. dentium,* were utilised. They were obtained from the culture collection CCUG. The bacteria were harvested after overnight culture on blood agar, washed, adjusted to identical concentration and UV-inactivated. In some experiments UV-inactivated bacteria were subjected to sonication, formalin fixation or treatment with protease or lysozyme before being used to stimulate blood cells.

S. pneumoniae and other streptococci of the viridans group (II, III)

The *S. pneumoniae* strains used in study II and III, include the virulent strain *S. pneumoniae* D39 (D39 WT), its pneumolysin-deficient mutant (D39 PLY-) and autolysin-deficient mutant (D39 AL-) (180, 195), as well as five isolates from children with acute *otitis media* and two isolates from the culture collection CCUG. In addition, in study II a non-capsulated *S. pneumoniae* strain from CCUG was used in the phagocytosis/bacterial killing assays, and in study III, the ROS-inducing potential of two clinical isolates with low hemolytic activity was tested. The pneumococcal isolates were serotyped by standard methods and selected strains were also analysed by multilocus sequence typing (MLST).

Pneumococci were cultured in broth to late log phase, in the presence or absence of the autolysin inhibitor choline chloride. The bacteria were washed, adjusted to the same concentration and inactivated by UV-light. After the process of freezing and thawing the preparations at least 80% of the autolysis-prone bacteria had autolysed, while the majority of the autolysis-inhibited bacteria remained intact. To separate and analyse the factors released by autolysis, autolysed bacteria were centrifuged at maximal speed, whereafter the supernatant was collected and the pellet resuspended in an equal volume of PBS. The supernatant was heated, treated with trypsin, lysozyme or cholesterol before its ability to stimulate blood cells was tested.

In study II, seven strains belonging to species in the *Streptococcus viridans* group were used. They were obtained from different culture collections (VPI, NCTC, CCUG and ATCC).

Purified bacterial components (I, III)

In study I, cells were stimulated with microbial components, including purified LPS, the purified Gram-positive cell wall components PG, LTA and MDP, isolated cell wall fragments and phosphopeptidomannan isolated from *C. albicans* or chitin/chitosan isolated from zygomycetal cell walls.

In study III native pneumolysin (PLY) (302) and modified pneumolysin (PdT), in which three amino acids have been substituted ($Asp_{385} \rightarrow Asn$, $Cys_{428} \rightarrow Gly$ and $Trp_{433} \rightarrow Phe$) leading to abolished cytolytic and complement activating abilities, were utilised (303). In some experiments, pneumolysin was treated with cholesterol before being added to the neutrophils.

Ovalbumin and OVA-expressing *E. coli* and lactobacilli/lactococci (VI)

Soluble OVA (Sigma) was purified from contaminating LPS before being used in the cell cultures.

E. coli expressing the entire chicken OVA protein was constructed by combining elements of the plasmids pOMP21 (304, 305) and pIAβ8 (306). OVA-expression was verified and quantified with Western blot and ELISA, respectively. To construct vectors producing OVA in Gram-positive bacteria, a synthetic gene was constructed. This gene encoded the amino acids 319-386 of chicken ovalbumin (OVA_f), but with codons optimised for usage in lactobacilli, was obtained (GeneScripts Corp., Piscataway, NJ, USA). The OVA-fragment, as well as a GFP-gene, with codons optimised for lactobacilli usage, were inserted into various expression plasmids functioning in lactobacilli, lactococci and *E. coli*. Two *Lactobacillus* strains, one *Lactococcus* and one *E. coli* strain were transformed with the vectors, and OVA-expression was verified by Western blot and GFP-expression by fluorescence scanning.

Isolation and activation of immune cells

Isolation and in vitro stimulation of human PBMC (I, II)

Peripheral blood mononuclear cells (PBMC) were prepared from blood-donor buffy coats by density gradient centrifugation. The cells were stimulated with UV-inactivated bifidobacteria (I), pneumococci or *Streptococcus viridans* bacteria (II). Supernatants were harvested after 24 h and 5 d, the latter time point to measure IFN-γ. In some experiments, the PI3K inhibitor Wortmannin, the p38 inhibitor SB 203580, the ERK inhibitor PD98059, the JNK inhibitor SP600125 or the IKK inhibitor VII were added 10 min before stimulation with bacteria.

Isolation of human neutrophils (III)

Polymorphonuclear cells, neutrophils, were isolated from blood-donor buffy coats. After dextran sedimentation and density gradient centrifugation, the pelleted cells were washed, and remaining red blood cells were lysed. Neutrophils were resuspended in Krebs-Ringer phosphate buffer and used for experiments within 6 h.

Isolation of murine cell populations (IV, Appendix I)

APC populations were isolated from Peyer´s patches, spleens or peritoneal cavity of BALB/c mice. Unfractionated Peyer´s patch cells, splenocytes or peritoneal cells, or purified populations thereof were used as APCs. $CD11c^+$ cells were enriched by magnetic adsorption cell sorting (MACS) and in some experiments cells were further purified by FACS-sorting. $CD11b^+$ and $CD11b^-$ peritoneal cells were separated using MACS-beads.

OVA-specific $CD4^+$ T cells were purified from the lymph nodes of OVA-TCR-transgenic BALB/c DO11.10 mice (307). The transgenic T cells were isolated by α-FITC conjugated MACS beads after staining of transgenic cells with FITC-conjugated KJ1-26 antibody (anti-DO11.10 TCR) (308).

Co-cultures of APC-T cells (IV, Appendix I)

Unfractionated suspensions of Peyer´s patches or spleen cells were pulsed overnight with bacteria or OVA, irradiated and washed before being mixed with T cells. Adherent peritoneal cells, as well as purified $CD11c^+$ cells, were pulsed with bacteria or OVA for 2 h in 96-well round bottom plates, centrifuged in the plates and washed three times with medium.

OVA-specific $CD4^+$ T cells were added to the antigen-pulsed APCs and after 4 days of co-culture the proliferative response was measured by [^{3}H] - thymidine incorporation and the levels of IFN-γ, IL-17, IL-10 and IL-13 were measured using DuoSet kits. In some experiments, the prostaglandin inhibitor indomethacin, the NO-inhibitor N^G-Monomethyl-L-arginine (MMA) or neutralising IL-10 or TGF-β or isotype-control antibodies were present both during pulsing of APCs and in the APC-T cell co-cultures.

Determination of cytokines and other mediators (I, II, IV)

Concentrations of human IL-12, TNF, IL-6, IL-8 and IL-10 in the 24 h PBMC supernatants and IFN-γ in the 5 d PBMC supernatant were measured by ELISA (I, II). In addition, in study I the intracellular production of IL-12 in monocytes was measured by flow cytometry, 22 h after stimulation of PBMC with FITC-stained *B. dentium* or *B. adolescentis*.

Murine cytokines (IL-6, IL-10, TNF and IL-12) were quantified, in the supernatants of 24 h cultures of APCs and bacteria, using the Cytometric Bead Array (CBA)

(BD Pharmingen). NO was immediately analysed in the 24 h co-cultures using Griess modified reagent (Sigma). PGE_2 was quantified using the PGE_2 EIA monoclonal kit (Cayman Chemical Company, Ann Arbor, MI, USA).

Phagocytosis and killing assays (I, II, IV)

Uptake of bacteria by human monocytes was analysed microscopically (I, II) and by flow cytometry using FITC-stained bacteria (I). Before the microscopical analysis, PBMC were incubated with UV-inactivated bacteria for 30 min, washed, cytocentrifuged and stained with Diff-quick. At least 60 monocytes were counted for the presence of surface-associated or internalized bacteria, and the average number of bacteria per monocyte was calculated (I, II).

In study II, we also analysed the ability of whole human blood to eliminate pneumococci. The non-capsulated *S. pneumoniae* 10708 was grown to early exponential phase in choline containing broth, and incubated at approximately 10^4 cfu with 1 ml of heparinised human blood. Viable counts were performed on the initial mixture and after 2-3 h of co-incubation. Viable counts were determined before and after triton lysis of blood cells. We tested how addition of UV-inactivated intact, or autolysed, pneumococci affected the ability of blood cells to eliminate viable pneumococci, and also tested if soluble factors, produced by PBMC in response to intact/autolysed bacteria, affected the ability of whole blood cells to eliminate viable pneumococci.

Phagocytosis of bacteria by murine APCs was analysed using *L. sakei* and *E. coli* expressing GFP or bacteria stained with the pH-sensitive dye pHrodo™ succinimidyl ester (SE) (Invitrogen), which yields colour only when present within an acidic compartment. APCs were incubated with fluorescent bacteria for 50 min at 37°C before being fixed in paraformaldehyde, stained with surface antibodies and analysed by flow cytometry.

Production of reactive oxygen species by neutrophils (III)

NADPH-oxidase activity was determined using isoluminol/luminol and chemiluminescence systems that allow determination of both extracellularly and intracellularly released reactive oxygen species (ROS) (309). For measuring total ROS, a cell-permeable horse radish peroxidase (HRP) and luminol were used. To measure only extracellular radicals, the non membrane permeable luminescence amplifier isoluminol was used instead of luminol, while measurements of intracellular ROS alone were performed in the presence of superoxide dismutase and catalase, which scavenge extracellular radicals. Bacteria or pneumolysin was added to neutrophil containing reaction tubes, and the light emission in the Biolumat apparatus was recorded continuously.

Calcium production by neutrophils (III)

For measurements of Ca^{2+} fluxes, neutrophils were loaded with Fura-2/AM and stimulated with pneumococci or pneumolysin in a fluorescence spectrophotometer. Fura-2 was excited with light of dual wavelengths (340 and 380 nm) and monitored for emission at 510 nm, and the calcium concentration in the stimulated neutrophils was determined as the 340 nm/380 nm emission ratio. Some experiments were performed in the presence of EGTA to determine the increase in cytosolic Ca^{2+} in the absence of an influx over the plasma membrane.

Viability assay (I, II, III)

The viability of human blood cells after stimulation with bacteria, pneumolysin or various inhibitors was assayed by flow cytometry after staining with the nucleic acid dye 7-amino-actinomycin D (7-AAD). The viability of PBMC was analysed after 24 h of stimulation (I, II), while the viability of neutrophils was analysed after 30 min of stimulation (III).

Statistical methods

The statistical methods used in analysing the data in the various studies include Wilcoxon matched pairs test, Mann-Whitney test, Student's t-test and paired Student's test.

RESULTS AND COMMENTS

Intact Gram-positive bacteria are potent inducers of phagocyte activating cytokines in human PBMC

Gram-positive bacteria must be intact in order to trigger IL-12 production in human monocytes (I)

Our group has previously shown that Gram-positive and Gram-negative bacteria induce different patterns of cytokines from human PBMC (103, 127, 128). The most prominent difference is that most Gram-positive bacteria are very potent inducers of IL-12 in human monocytes, while Gram-negative bacteria induce little IL-12 in unstimulated monocytes (103). In study I, we used bifidobacteria as a model organism to study the molecular background to the strong ability of the Gram-positive bacteria to induce IL-12 production in human monocytes. At first, the microbial structures inducing IL-12 production were investigated. Bifidobacteria were UV-inactivated, formalin fixed, sonicated or treated with heat, lysozyme or protease. Thereafter, the various bacterial preparations were examined microscopically and their abilities to induce IL-12 production in PBMC were tested. We noted that only preparation containing intact bacterial cells were able to induce IL-12 production. As a comparison, we also tested the effect of the various treatments on IL-6 production, and noted that an intact cell wall structure was not needed for IL-6 induction. In accordance with the inability of bacterial sonicates to induce IL-12 production, isolated cell wall components, such as peptidoglycan, MDP or LTA did not induce IL-12 production from the monocytes. LPS induced some IL-12 production (ten times less than Gram-positive bacteria), but only if the monocytes had been pre-treated with IFN-γ.

Autolysis of *S. pneumoniae* inhibits production of phagocyte activating cytokines in human PBMC (II)

S. pneumoniae is a Gram-positive pathogen with a peculiar tendency to spontaneously decompose, when reaching the stationary phase of growth. This is mediated by enzymes called autolysins, the major autolysin being LytA (190). The ability to undergo autolysis adds to the virulence of pneumococci for unknown reasons. As we had noted that Gram-positive bacteria needed to present an intact cell wall to induce IL-12 production in human monocytes, we speculated that pneumococcal autolysis would serve to avoid triggering of IL-12 production. We compared the cytokines produced in PBMC in response to seven pneumococcal strains of various sero- and genotypes with the cytokines produced in response to strains from related *Streptococcus* species belonging to the viridans group. These are genetically closely related to pneumococci, but do not undergo spontaneous

autolysis. Indeed, none of the *S. pneumoniae* strains induced detectable production of IL-12 in PBMC, while all viridans streptococci induced very high levels of IL-12 (1-5 ng/ml). We also noted that *S. pneumoniae* induced 10-50 times lower levels of the phagocyte activating cytokines TNF and IFN- γ in PBMC than related viridans streptococci, while all tested bacteria induced similar production of IL-6, IL-8 and IL-10.

To investigate whether spontaneous autolysis was responsible for the selective failure of pneumococci to induce IL-12 and phagocyte activating cytokines, seven *S. pneumoniae* isolates were cultured in broth to stationary phase either in the presence or absence of the autolysin inhibitor choline, before being used to stimulate blood cells. Pneumococci with inhibited autolysins induced significantly more TNF, IFN- γ and IL-12 than pneumococci cultured under standard conditions. Production of IL-6, IL-8 and IL-10 was, in contrast, similar regardless of whether the bacteria could autolyse or not.

We concluded that autolysis surely reduced the ability of pneumococci to trigger phagocyte activating cytokines. This was confirmed by comparing the cytokine pattern induced by the LytA- negative mutant (D39 AL-) and its WT control (D39 WT). As expected, the D39 AL- mutant induced significantly more TNF and IFN-γ production than D39 WT and again IL-6, IL-8 and IL-10 were equally well induced by lysed and intact pneumococci. Choline had no effect on the IL-12 inducing ability of *S. mitis* and did not affect monocyte viability.

Bacterial fragments inhibit production of phagocyte activating cytokines in response to intact bacteria (I, II).

Intact Gram-positive bacteria, but not their fragments, induced IL-12. In study I we demonstrated that bacterial fragments and isolated components of the Gram-positive cell wall, such as PG, LTA and MDP also inhibited IL-12 production in response to intact bacteria. Thus, if blood cells were incubated with sonicated bacteria or isolated cell wall components prior to stimulation with intact bifidobacteria, the IL-12 production was dose-dependently inhibited. In contrast, the ability of bifidobacteria to induce IL-6 production was unaffected by preincubation with fragmented bacteria.

The same phenomenon was noted in study II. Although the preparations of autolysin-positive pneumococcal strains contained up to 20 % intact bacterial structures when examined in the microscope, the were profoundly inefficient in triggering the phagocyte activating cytokines TNF and IFN-γ, even if used in a ten-times higher dose than intact pneumococci. Thus, the presence of bacterial degradation products appeared to inhibit the production of TNF and IFN-γ which otherwise would be induced by intact bacteria. To test this, PBMC were stimulated with intact pneumococci in the presence of various concentrations of the

corresponding autolysed bacteria. Indeed, the presence of autolysed bacteria reduced the production of TNF, IFN-γ and IL-12 in response to intact bacteria alone, in a dose-dependent manner. Significant inhibition of TNF ($p < 0.05$) and IFN-γ ($p < 0.01$) responses were obtained by admixture of a ten-fold lower dose of autolysed bacteria compared to intact bacteria. Thus, if only one bacteria in ten autolyses, the TNF and IFN-γ responses to the remaining intact bacteria is significantly reduced. In contrast to the pronounced inhibition of phagocyte activating cytokines by bacterial fragments, production of IL-6, IL-8 and IL-10 was unaffected or slightly increased when autolysed bacteria were admixed with intact pneumococci, which is expected, since fragmented bacteria can induce these cytokines.

The preparations of wild type pneumococci grown in broth under normal conditions to stationary phase, supposedly contained both large and small fragments of bacterial cell walls. Stationary phase grown *S. pneumoniae* were separated into a soluble fraction and a pellet by centrifugation, and these fractions were admixed with intact pneumococci and PBMC, whereafter cytokine production was measured. Both the soluble and pellet fractions reduced TNF and IFN-γ production in response to intact bacteria, but the effect was most pronounced when the supernatant fraction was added. We tested whether fragments of streptococci generated by sonication would also down-regulate these cytokines in response to intact bacteria. *S. mitis*, which is not capable of autolysis, was subjected to sonication and PBMC were stimulated with intact UV-inactivated *S. mitis* in the presence or absence of various doses of sonicated *S. mitis.* A dose-dependent inhibition of TNF, IFN-γ and IL-12, i.e. cytokines yielding phagocyte activation, but not of IL-6, IL-8 or IL-10, was seen when mixing intact and sonicated *S. mitis*.

Furthermore, purified PG, MDP and LTA, i.e. components of the Gram-positive cell wall, could inhibit TNF, IFN- γ and IL-12 production induced by intact pneumococci or *S. mitis*, which confirms and extends our findings that IL-12 production by monocytes in response to bifidobacteria is down-regulated by bacterial sonicates as well as soluble cell wall components.

Requirement of phagocytosis for production of phagocyte activating cytokines in PBMC (I, II)

Particles are phagocytosed, whereas soluble components enter the phagocyte via pinocytosis. We hypothesized that bacterial induced IL-12, but not IL-6, IL-8 or IL-10, was dependant on phagocytosis. In study I, PBMC were incubated with cytochalasin, which inhibits actin-polymerisation, before being stimulated with bifidobacteria. This resulted in a dose-dependent inhibition of IL-12, but not IL-6, production in response to intact Gram-positive bacteria. Thus in response to Gram-positive bacteria IL-12, but not IL-6, appeared to depend on phagocytosis and/or rearrangements of the cytoskeleton. Using flow cytometry we examined whether

intracellular IL-12 was present in human monocytes that had phagocytosed GFP-stained bifidobacteria. Only monocytes that harboured bacteria produced IL-12, though only a fraction of the monocytes staining positive for bacteria produced IL-12. Although we could not formally prove that the bacteria were intracellular, examination of cytospin slides revealed that bacteria were, indeed, present within monocytes.

In contrast, LPS induced IL-12 in IFN-γ preincubated PBMC is not affected by cytochalasin treatment (310). Thus, LPS most likely induces IL-12 production via signalling from surface receptors, such as TLR4. In contrast, bifidobacteria exclusively induced signals resulting in IL-12 production after cytoskeleton rearrangements, maybe by interacting with receptors recruited to phagosomes, such as NOD2 and TLR2.

Further support for the role of phagocytosis in the induction of phagocyte activating cytokines comes from study II. We here noted that although intact pneumococci induced more IFN-γ, TNF and IL-12 than their autolysed counterparts, they still induced slightly lower levels of IFN-γ, 5 times less TNF and 20 times lower levels of IL-12 than related streptococcal species. Virulent *S. pneumoniae* strains contain a thick polysaccharide capsules that prevents phagocytosis and is a major virulence determinant in pneumococci. Such capsules are not produced by other related species in the viridans group, which are generally non-pathogenic. We speculated that the pneumococcal capsules sluggish production of TNF and IL-12 in response to pneumococci. A non-encapsulated *S. pneumoniae* strain (CCUG 10708) was grown in the presence of choline, and its ability to trigger cytokine production in PBMC was tested. This strain induced higher levels if TNF and IL-12 than any of the encapsulated pneumococcal strains tested. It induced similar levels of TNF as the viridans streptococci, but approximately 5 times less IL-12.

Involvement of PI3K, JNK and NFκB signalling for production of phagocyte activating cytokines in PBMC (I, II)

Phagocytosis of Gram-positive bacteria appeared to be important for induction of IL-12, and probably also TNF, by monocytes in response to these bacteria. We wondered if intracellular signalling pathways could be identified that were induced by Gram-positive bacteria in monocytes, resulting in production of phagocyte activating cytokines. In study I, PBMCs were incubated with inhibitors of the NF-κB, JNK, p38 or PI3K pathways, before stimulation with bifidobacteria or, as a comparison, LPS/IFN-γ. After 24 h of culture the amounts of IL-12 and IL-6 in the culture supernatants were determined. Strikingly, inhibition of PI3K with wortmannin had opposite effects on the IL-12 production induced by bifidobacteria as compared to LPS/IFN-γ stimulation, and inhibited IL-12 in response to bifidobacteria, but augmented LPS-induced IL-12. The PI3K is involved in both phagocytosis and phagosome maturation (88-90). Thus, the fact that inhibition of

the PI3K prevented IL-12 response to bifidobacteria is in line with the assumption that phagocytosis of Gram-positive bacteria is necessary for IL-12 production, as the receptors recognising the bacteria were probably either present in the phagosome or recruited during the phagosomal maturation process. In sharp contrast, PI3K blocking permitted LPS, which normally is a very poor inducer of IL-12 production in human PBMC, to induce levels of IL-12 similar to those induced by intact Gram-positive bacteria. Similarly, production of IL-12 in response to intact *E. coli* was strongly increased upon PI3K-inhibition, as was the TNF production in response to soluble components of the Gram-positive cell wall (data not shown). Thus, in the absence of PI3K activation, Gram-negative bacteria, as well as soluble bacterial components may trigger cell surface receptors leading to IL-12 production. In contrast to its profound effect on IL-12 production, production of IL-6 was not affected by PI3K-inhibition.

Inhibition of the MAPK JNK and p38 and NF-κB pathways, diminished the production of IL-12 induced by Gram-positive bacteria. However, inhibition of JNK and p38 differed in that p38 also was of chief importance for IL-6 induction, while JNK activation appeared to be dispensable for induction of IL-6. As p38 has been shown to play a role in phagocytosis (311), this may be the reason for the reduced IL-12 production upon its inhibition. NF-κB is activation by various stimuli, and signalling through JNK has been shown to activate NF-κB in addition to AP-1 upon stimulation with Group B Streptococci (311).

In study II we had seen that the phagocyte activating cytokines IL-12, TNF and IFN-γ were selectively induced by intact Gram-positive bacteria, while cell wall fragments instead inhibited production of these molecules. We speculated that a common intracellular signalling pathway would induce production of TNF and IL-12, but not IL-6, IL-8 and IL-10 in monocytes. In search of such a pathway, selectively promoting phagocyte activating cytokines, we blocked various signalling molecules in PBMC and thereafter stimulated the cells with intact *S. pneumoniae* or *S. mitis*. When the JNK-pathway was blocked, production of TNF, IFN- γ and IL-12 was strongly reduced, while production of IL-6, IL-8 and IL-10 remained intact. Thus, a possibility would be that the JNK-pathway is selectively induced by intact Gram-positive bacteria, but not bacterial fragments, which instead might inhibit induction of this pathway.

Autolysed pneumococci inhibit phagocytosis (II)

Phagocytosis and subsequent decomposition, is the major mechanism to kill bacteria. We tested whether fragments of the pneumococcal cell wall, released during autolysis, could block interactions between intact bacteria and monocytes. PBMC were incubated with intact pneumococci in the presence or absence of autolysed bacteria for 30 min, after which they were spun onto glass slides, stained and examined microscopically. Pneumococci prevented from autolysing bound in chains to the monocyte surface, but were not internalised after 30 min incubation,

reflecting the resistance of encapsulated pneumococci to uptake by phagocytes in the absence of capsule-specific antibodies. Preparations in which the pneumococci were allowed to autolyse showed negligible interactions of the remaining intact bacteria with monocytes. Further, admixture of autolysed bacteria to intact bacteria significantly decreased the number of intact bacteria interacting with monocytes. As encapsulated virulent pneumococci were not phagocytosed under the conditions employed, we used a capsule-negative pneumococcal strain (CCUG 10708) to study the effect of autolysed bacteria on phagocytosis. In contrast to the encapsulated strains, the non-encapsulated 10708 strain was frequently seen inside the monocytes, within vacuoles. When equal amounts of intact and autolysed *S. pneumoniae* 10708 were added to PBMC, the presence of the latter significantly decreases phagocytosis of intact bacteria.

Phagocytosis is the process by which the particle becomes engulfed, ending up in a phagosome. For killing and decomposition of bacteria, a number of bactericidal component need to be recruited to the phagosome in a process termed phagosome maturation. Killing of non-encapsulated pneumococci by blood cells, in the absence and presence of autolysed pneumococci, was assessed by viable counts. A low dose of live bacteria was added to heparinised blood and remaining viable bacteria were enumerated after 2-3 h of incubation. The viable strain used was prevented from autolysis by culture in choline. Admixture of UV-inactivated intact *S. pneumoniae* D39 AL-, a mutant that does not spontaneously autolyse, did not affect the rate of bacterial elimination. However, when the same amount of UV-inactivated autolysed D39 WT was admixed, almost ten times more viable pneumococci remained extracellularly. Thus, the presence of autolysed, but not inactivated intact bacteria, decreased the ability of blood cells to eliminate viable pneumococci. To address whether large or small bacterial fragments were responsible for inhibiting bacterial uptake, the supernatant and pellet fractions of autolysed *S. pneumoniae* D39 were separated by centrifugation and their effects on elimination of viable bacteria was tested. The supernatant fraction of autolysed bacteria did not affect bacterial uptake, while the pellet fraction strongly inhibited the elimination of viable bacteria. Thus, large but not small bacterial fragments appeared to prevent phagocytosis. Similarly, in study I we noted that neither PG nor LTA affected the monocytic uptake of bifidobacteria. Thus, small bacterial fragments inhibit production of phagocyte activating cytokines by other means than inhibition of phagocytosis. On the other hand, large bacterial fragments inhibit phagocytosis, which could be the reason for their effect on cytokine production.

The phagocyte activating cytokines produced in response to UV-inactivated intact pneumococci increase the ability of blood cells to kill viable bacteria (II).

The PBMC supernatant after stimulation with intact pneumococci contained much more TNF and IFN- γ than after stimulation with autolysed pneumococci or a

mixture of intact and autolysed bacteria. Since TNF and IFN-γ have been shown to contribute to bactericidal killing by stimulating NO synthesis and phagosome maturation (312-314), we wanted to test if these supernatants differed in ability to activate phagocyte killing. Thus, non-encapsulated *S. pneumoniae* 10708 was incubated with heparinised human blood in the presence or absence of 10 µl supernatant obtained after 5 days stimulation of PBMC with either UV-inactivated intact pneumococci, the soluble fraction of autolysed pneumococci or a mixture of intact pneumococci and the soluble fraction of autolysed pneumococci. The presence of PBMC supernatant did not affect this number of extracellular bacteria remaining after 2 h of incubation with blood. However, viable counts after lysis of blood cells (i.e. measuring both intra- and extracellular viable bacteria) showed that the presence of supernatant from PBMC stimulated with intact pneumococci, but not the soluble fraction of autolysed bacteria or a mixture of intact and autolysed bacteria reduced the number of live bacteria 5 times. Since there were no differences in the amount of extracellular bacteria, this most likely reflects an increased intracellular killing. Thus, indeed the supernatant with the highest content of TNF and IFN-γ was most efficient in stimulated phagocyte killing.

Pneumolysin released during *S. pneumoniae* autolysis activates the neutrophil NADPH-oxidase

Autolysed, but not intact pneumococci, induces ROS-production in human neutrophils (III)

After phagocytosis, bacteria are killed by a combination of oxygen-dependent and independent mechanisms. In neutrophils the most important microbicidal mechanism is to produce oxygen-derived radicals into the phagosome. These radicals are toxic to the bacteria. The process of radical production is initiated by assembly of a multicomponent enzyme complex called the NADPH-oxidase. If this complex is formed at the membrane of an intracellular compartment, the resulting ROS-production will be intracellular. For unknown physiological reasons, the NADPH-oxidase can also be formed at the cell membrane, which will result in extracellular radical production. As neutrophils are recruited to the site of pneumococcal infections and are central in the elimination of pneumococci, we investigated the ability of neutrophils to produce oxygen radicals in response to pneumococci which were capable or incapable of undergoing autolysis. To our surprise, we noted that much higher radical production was produced in response to autolysed than intact pneumococci. We investigated which components that were involved in triggering this pronounced ROS-production, and found that the ROS-inducing factor was confined to the supernatant of spontaneously lysed pneumococci. Thus, autolysis of pneumococci was required to release the ROS-inducing factor. When pneumococci were cultured in the presence of choline to prevent autolysis, their ability to trigger the NADPH-oxidase was strongly reduced.

Further, the LytA deficient pneumococcal strain D39 AL- was much less active in stimulating ROS-production, compared to its otherwise isogenic wild type control.

Pneumolysin released during autolysis is the ROS-inducing agent (III)

In order to characterise the factor released during pneumococcal autolysis that induced ROS-production, we treated the supernatant of *S. pneumoniae* A17 with heat or degrading enzymes. Treatment of pneumococcal supernatant with heat or trypsin completely abrogated its ability to activate neutrophils, whereas treatment with lysozyme, which degrades peptidoglycan backbone, had no effect on the chemiluminescence response. Thus, in addition to being released upon autolysis, the factor inducing oxygen radicals is of a proteinaceous nature.

We hypothesized that pneumolysin was the neutrophil activating factor, since it is an intracellular toxoid protein known to be released during autolysis (171). We tested the capacity of a pneumolysin-deficient strain of *S. pneumoniae*, D39 PLY- (which still has an intact autolysin), to activate the neutrophil NADPH-oxidase. Preparations of D39 PLY- bacteria lacked neutrophil stimulating activity, regardless of whether they were grown in the presence or absence of choline. Western blot analysis confirmed that pneumolysin was present in the supernatants of stationary phase grown wild type pneumococci, but not in the supernatant of the PLY- mutant. Next, highly purified pneumolysin was added to neutrophils and the chemiluminescence response was monitored. Purified pneumolysin was highly effective in inducing neutrophil NADPH-oxidase activation, and concentrations down to 1 ng/ml induced measurable responses. Optimal doses (50 ng/ml) induced an oxidative response, comparable in magnitude to PMA, which is one of the most potent ROS-inducing agents known. With doses higher than 100 ng/ml pneumolysin, the response again decreased, probably due to cell-toxic effects.

Pneumolysin must interact with cell membrane cholesterol and needs a functional cytolytic unit in order to activate ROS-production (III).

Pneumolysin binds to cholesterol in eukaryotic cell membranes and can, when present in high concentrations, form membrane pores. Addition of cholesterol to the supernatant of spontaneously autolysed *S. pneumoniae* A17 or to purified pneumolysin, dose-dependently inhibited generation of ROS and a high concentration of cholesterol completely blocked ROS formation. Cholesterol did however not affect ROS-generation by the chemoattractant fMLF. This suggests that interaction of pneumolysin with membrane cholesterol is of importance for its stimulation of the NADPH-oxidase and generation of reactive oxygen radicals.

A modified pneumolysin (PdT) in which three amino acids have been substituted ($Asp_{385} \rightarrow Asn$, $Cys_{428} \rightarrow Gly$ and $Trp_{433} \rightarrow Phe$) lacks both the cytolytic activity

and the ability to activate the complement system (303, 315). We tested this mutated pneumolysin in our system, and found that it did not induce any NADPH-oxidase activity at concentrations up to 100 ng/ml when incubated with neutrophils. Pneumolysin is an important virulence determinant for pneumococci, and is present in virtually all clinical isolates. However, a number of clinical *S. pneumoniae* isolates, containing mutated variants of pneumolysin with reduced cytolytic activity, have been characterised. We tested the abilities of two such isolates, expressing pneumolysins with reduced cytolytic activities (187), to induce activation of the neutrophil NADPH-oxidase. None, of these isolates induced significant amounts of ROS from neutrophils, showing that an intact cytolytic unit is needed for efficient induction of ROS-production.

Pneumolysin induces ROS production into intracellular compartments and the induction is dependent on influx of extracellular calcium (III).

The NADPH-oxidase may assemble on the plasma membrane or on membranes lining intracellular compartments, such as the phagsome, granulae etc. As a consequence, oxygen radicals may be generated extracellularly, intracellularly of both. We employed the luminol/isoluminol method to discriminate between intra- and extracellularly-generated superoxide (309). We found that in response to pneumolysin, the major fraction of ROS formed was generated intracellularly and only relatively small amounts of superoxide were secreted.

Activation of the NADPH-oxidase is often coupled to a rise in the intracellular calcium concentration, and we tested if this was the case upon pneumolysin stimulation. Stimulation of neutrophils with pneumococcal supernatant or with native pneumolysin, but not cytolytic deficient pneumolysin, induced a rise in the intracellular calcium concentration in neutrophils. This rise was dependent on the presence of extracellular Ca^{2+} and the pneumolysin-induced neutrophil NADPH-oxidase activity was abolished in Ca^{2+}-free medium.

Responses of neutrophils to unrelated stimuli are altered after pneumolysin stimulation (III).

We asked whether interaction with pneumolysin would alter the functional response of neutrophils to subsequent stimulation with unrelated stimuli. Neutrophils were preincubated (30 min at 37^{o}C) with native pneumolysin (PLY) (50 ng/ml) or, as a control, mutant pneumolysin (PdT). Thereafter a ROS response was stimulated with the chemotactic peptide fMLF or the phorbol ester PMA. Pretreatment of neutrophils with pneumolysin resulted in a prolonged response to fMLF. This was not seen using pretreatment with mutant pneumolysin. The subsequent response to PMA was instead strongly reduced in neutrophils pre-treated with pneumolysin. Thus, the interaction of haemolytic active pneumolysin with neutrophils led to alterations in their normal subsequent reactions patterns.

One may speculate that the altered responsiveness leads to decreased functional capacity of the neutrophils to combat viable bacteria, but further experiments are needed to investigate this hypothesis.

Gram-negative bacteria are more efficient activators of $CD4^+$ T cells than Gram-positive bacteria or soluble antigens

$CD4^+$ T cell responses induced by APCs from the spleen

Gram-negative bacteria activate $CD4^+$ T cells more efficiently than Gram-positive bacteria (IV)

In study I and II we confirmed that intact Gram-positive bacteria are potent inducers of IL-12 in human PBMC. IL-12 is an important factor activating $CD4^+$ T cells to mature to Th1 polarised IFN-γ producing cells. In study IV we wanted to investigate the degree and type of $CD4^+$ T cell activation that was induced by different antigen-presenting cells (APCs) that had been stimulated with Gram-positive or Gram-negative bacteria. Thus, the $CD4^+$ T cell response induced by a soluble antigen should be compared to the $CD4^+$ T cell response to the same antigen when presented to the APC as a constituent of a Gram-positive or Gram-negative bacterial cell. To enable this comparison, a model system was established were OVA-producing bacteria or soluble LPS-free OVA was presented to DO11.10 OVA-transgenic $CD4^+$ T cells by different APC subsets. Promoters and codon usage differ between Gram-positive and Gram-negative bacteria and it was necessary to construct novel plasmids that could generate OVA-production in Gram-positive bacteria. A synthetic gene was constructed, encoding an OVA-fragment, by using codons adapted to function in Gram-positive bacteria. This gene was cloned into different expression plasmids known to function in lactobacilli. As a control antigen, not recognised by DO11.10 cells, GFP was used. This also generated fluorescent bacteria which could be used to trace interaction with APCs. The plasmid encoding almost the entire OVA-protein was inserted into *E. coli*, while expression plasmids encoding the OVA-fragment 319-386 (OVA_f) were introduced into *E. coli*, *Lactobacillus sakei*, *Lactobacillus plantarum* and *Lactococcus lactis*. Using western blots, we verified that all strains expressed OVA, and noted that OVA_f generated approximately 10 times higher peptide levels in the lactobacilli, than in the lactococcus and *E. coli*. For the *E. coli* expressing the entire OVA-protein, the amount expressed was quantified by ELISA and 5×10^7 bacteria contained 0.19 µg OVA. Both the OVA-fragments and the entire OVA was produced only intracellularly. All bacteria were UV-inactivated before being used in the *in vitro* cultures.

In initial experiments, we used unfractionated splenocytes to compare the efficacy of soluble OVA and OVA expressed in *E. coli* to induce $CD4^+$ T cell proliferation. Since a low grade of proliferation was induced also to bacteria not expressing OVA, the OVA-specific proliferation, Δ prol, was defined as the difference in proliferative response using OVA-producing and empty control-bacteria. Optimal concentrations of *E. coli*, 5×10^7 /ml, induced an OVA-specific proliferation, Δ prol, of 10,000 cmp. A similar proliferative response was obtained by stimulation with 100 µg/ml of soluble OVA. As 5×10^7 *E. coli* contained 0.19 µg OVA, approximately 500 times more soluble than bacterial OVA was needed to induce the same magnitude of OVA-specific T cell proliferation.

Next, we compared the abilities of OVA expressed within a Gram-positive and Gram-negative bacterial cell to activate OVA-specific $CD4^+$ T cells. Also here we used unfractionated spleen cells as APCs. Although the lactobacilli and lactococci were potent producers of OVA, they induced a much lower grade of T cell proliferation than did OVA-expressing *E. coli* strains. In fact, only *L. sakei*, of the OVA-expressing Gram-positive strains tested, induced a proliferation that was significantly higher than that induced by its non-OVA-expressing control, according to Student's paired t-test (Δ prol = 600 cpm for 5×10^6 bacteria/ml). Thus, in optimal doses, the Δ proliferation to *E.* coli-OVA was at least 15 times higher than the Δ proliferation to lactobacilli/lactococci-OVA. Of note, the optimal dose of *L. sakei* was 5×10^6/ml, while for *E. coli* the optimal dose was 5×10^7/ml.

$CD11c^+$ dendritic cells are regarded as the most efficient APCs, $CD11c^+$ cells from the spleen were enriched using α-CD11c-coated magnetic beads, pulsed with soluble or bacterial OVA, washed in the plates and co-cultured with OVA-specific Tg T cells. Similarly to the unfractionated cells, the CD11c-enriched cells supported a strong OVA-specific T cell proliferation to *E. coli*-OVA (Δ prol = 18,000 cpm), but only a week proliferation to *L. sakei*-OVA (Δ prol = 800 cpm). Furthermore the CD11c-enriched splenocytes induced a strong T cell proliferation to 100 µg/ml soluble OVA (25,000 cpm), and a modest T cell proliferation to 10 µg/ml soluble OVA (2,500 cpm). Thus, the data suggests that this cell population is responsible for the APC activity of whole spleen cells.

As the MACS-separation only yielded a semi-purified DC population, the $CD11c^+B220^-$ DCs were purified by FACS-sorting and used as APCs. These cells efficiently induced T cell proliferation to *E. coli*-OVA (Δ prol = 14,000 cpm) and soluble OVA (8,000 cpm), though we could not detect OVA-specific T cell proliferation to *L. sakei*-OVA.

Splenocytes pulsed with bacterial and soluble OVA induce different T cell polarisation (IV)

To investigate whether soluble, "Gram-positive" or "Gram-negative" OVA induced different T cell polarisation, we quantified IFN-γ, IL-13, IL-17 and IL-10 in the supernatants of APC-T cell co-cultures after 4 days. Similar cytokine patterns were found when unfractionated and CD11c-purified spleen cells were used as APCs. *L. sakei*–OVA tended to induce a modest OVA-specific IFN-γ response, but only low levels of the other T cell cytokines, in accordance with the modest T cell proliferative response. *E. coli*-OVA induced high levels of IFN-γ and IL-13. Modest levels of IL-17 and IL-10 were also detected, but the IL-10 was probably produced by monocytes and related cells, as IL-10 in similar amount was produced in bacteria stimulated cultures without T cells. The cytokine response to soluble OVA was dominated by high levels of IL-13, and only modest levels of IFN-γ, IL-17 and IL-10. Thus, bacterial presentation of OVA induced more IFN-γ production in the co-cultures than soluble OVA, which elicited a response dominated by IL-13 production when presented by spleen APCs. IL-13 is commonly considered to be a Th2 polarised cytokine.

CD4+ T cell responses induced by APCs from the peritoneal cavity

Adherent peritoneal cells activate T cells to *L. sakei*-OVA and soluble OVA, but not *E. coli*-OVA (IV)

The splenic APCs were poor presenters of Gram-positive antigens. We speculated that release of OVA required digestion of the thick Gram-positive cell wall, which might be a difficult task for conventional DCs, as they do not posses much bacterolytic enzymes. Resident peritoneal cells, which contain a high fraction of macrophages, might therefore be more suited for presentation of Gram-positive antigens. Peritoneal cells were harvested, purified by adherence to plastic and pulsed for 2 h with *L. sakei*-OVA, *E. coli*-OVA "empty" control-bacteria or soluble OVA. They were then washed and co-cultured with OVA-specific $CD4^+$ T cells. Peritoneal cells supported a weak, but statistically significant, OVA-specific T cell proliferation when stimulated with *L. sakei*-OVA (Δ prol = 550 cpm). To our surprise, no T cell proliferation, neither antigen-specific nor mitogen-induced (to control-bacteria), was detected in response to *E. coli*-OVA. In contrast, adherent peritoneal cells were efficient presenters of soluble OVA, both in the 100 µg/ml and 10 µg/ml dose, inducing T cell proliferations of 30,000 cmp and 20,000 cpm, respectively.

$CD11c^+$ cells from the peritoneal cavity efficiently present bacterial and soluble OVA to T cells (IV)

When adherent peritoneal cells (washed in 37°C medium after 2h of adherence to plastic) were analysed by FACS, we noted that in addition to a large population of macrophages ($CD11b^{high}CD11c^-F4/80^{high}SSC^{high}$), there was a DC-like population ($CD11c^+MHCII^+F4/80^{low}SSC^{low}$) constituting approximately 2% of the cells. We asked whether they were responsible for the antigen-presenting capacity of the adherent peritoneal cells. To test this, the peritoneal cells were separated into a CD11c-positive and a CD11c-negative fraction using MACS-beads.

The CD11c-negative cells were adhered to plastic, pulsed with antigen and co-cultured with T cells. This fraction had no capacity to activate T cells to *L. sakei*-OVA, and the capacity to present soluble OVA was at least 10 times lower compared to the unfractionated population. On the other hand, the $CD11c^+$ peritoneal cells were efficient APCs, inducing OVA-specific T cell proliferation not only to *L. sakei*-OVA (Δ prol.=1,500 cpm) and soluble OVA (75,000 cpm to 100 µg/ml and 20,000 cpm to 10 µg/ml), but also to *E. coli*-OVA. The proliferative response to *E. coli*-OVA (30,000 cpm/ml) was, similarly to when splenocytes were used as APCs, approximately fifteen times higher than the proliferative response to "Gram-positive" OVA. We concluded that the $CD11c^+$ cells present in the peritoneal cavity had a similar or even higher capacity than the $CD11c^+$ cells in the spleen, to present bacterial and soluble antigens in a manner resulting in T cell proliferation. However, their strong capacity to present a Gram-negative bacterial antigen was masked in the presence of peritoneal macrophages.

T cells activated with peritoneal APCs produce more IL-17 and IL-10 and less IL-13 and IFN-γ compared to T cells activated with splenic APCs (IV)

We compared the T cell polarisation induced by peritoneal APCs and splenic APCs by investigating the T cell cytokines produced in the co-cultures. *L. sakei*-OVA did not induce significant OVA-specific cytokine production, though there was a tendency of OVA-specific IL-10. Further, some IL-17 and IFN-γ was produced in a non-OVA-specific manner in response to *L. sakei*. Thus, equally high amounts of IL-17 and IFN-γ were produced to *L. sakei* with and without the OVA-plasmid, suggesting that NKT cells or NK cells could be the producers of these cytokines in response to innate recognition of Gram-positive bacteria.

$CD11c^+$ peritoneal cells pulsed with *E. coli*-OVA supported production of large amounts of T cell cytokines. IL-17, IL-10 and IL-13 were produced in significantly higher levels in response to *E. coli*-OVA than to *E. coli*-control, and there was also a tendency of a modest OVA-specific IFN-γ production. As splenic APCs induced high levels of mainly IFN- γ and IL-13, the tendency was that the DCs in the

peritoneal cavity induced production of more IL-17 and IL-10 and less IFN- γ and IL-13 from T cells.

In response to soluble OVA, the major observation was that the peritoneal APCs supported production of significantly less IL-13 from T cells than did splenic APCs. In conclusion, the antigen-presenting cell population in the peritoneal cavity may skew the T cell polarisation away from both Th1 and Th2 and favour induction of IL-17 and IL-10 producing cells.

Peritoneal macrophages inhibit T cell activation (IV)

Evidently, the peritoneal lavage contained a population of $CD11c^+$ MHC II^+ cells with high ability to present bacterial and soluble antigens to $CD4^+$ T cells. However, the presence of CD11c-negative macrophages shut down T cell proliferation to *E. coli*-antigens. We examined the role of macrophages in modulating antigen presentation of Gram-positive, Gram-negative and soluble OVA by adding back graded doses of peritoneal macrophages to the $CD11c^+$ purified peritoneal cells and using the mixture to present antigen to DO11.10 T cells. As expected, T cell proliferation and production of T cell cytokines in response to *E. coli*-OVA was dose-dependently inhibited by addition of CD11c-negative macrophages. High doses of macrophages also inhibited the *L. sakei*-OVA induced T cell activation, though low doses of CD11c-negative peritoneal cells instead seemed to potentiate the OVA-non-specific production of IL-17 and IFN-γ.

Addition of macrophages to the DC-T cell co-cultures only marginally affected T cell activation to soluble OVA. We speculated that bacteria, but not simple proteins, induced production of inhibitory substances from peritoneal macrophages. To test this hypothesis, supernatants from unstimulated, or bacteria stimulated, macrophage cultures were added to the co-cultures of antigen-pulsed $CD11c^+$ peritoneal cells and T cells. Indeed the supernatants from bacteria stimulated cultures inhibited T cell activation, while supernatant from unstimulated cultures had no gross effect.

Apparently soluble substances produced by peritoneal macrophages in response to bacteria counteracted T cell activation. Candidate factors for having this effect include IL-10, PGE_2 and NO, which are all produced in bacteria stimulated macrophage cultures. Also TGF-β, which has a well documented effect on inhibiting T cell responses, may be produced by peritoneal cells (266). We tested to inhibit the PGE_2 and NO production, or neutralise IL-10 and TGF-β in macrophage containing DC-T cell co-cultures. Inhibition of all these factors led to an increased T cell proliferation, though the increase was greater when PGE_2, IL-10 or TGF-β was inhibited, compared to inhibition of NO. Thus, all these mediators could potentially be involved in limiting T cell proliferation. We also studied how factors produced by macrophages affected the production of T cell cytokines in the

inhibited cultures, the individual factors appeared to affect the polarisation differently.

The observation that T cells stimulated with peritoneal DCs appeared to be more polarised towards Th17 and IL-10 production, and less polarised towards Th1 and Th2, might be explained by the presence of different levels of mediators in the two tissues. We suggest that TGF-β may be involved in limiting Th2 polarisation, IL-10, TGF-β and PGE_2 may all limit Th1 polarisation, PGE_2, IL-6 and IL-1β may promote Th17 polarisation, while NO inhibits the same and IL-10 may promote IL-10 producing T cells.

T cell stimulatory effects of APC subsets in Peyer´s patches, spleen and the peritoneal cavity

Characterisation of $CD11c^+B220^-$ cells in different compartments (Appendix I)

The $CD11c^+$ cells in the spleen, as well as in the peritoneal cavity, appeared to be the chief presenters of both bacterial and soluble antigens. However, the T cell activation and polarisation pattern induced by the $CD11c^+$ APCs from the two compartments differed in that the peritoneal APCs induced higher T cell proliferation and more IL-17 and IL-10, but less IL-13, in the co-cultures, than the splenic APCs.

We speculated that these differences in part could depend on the different types of APCs present within the two compartments. In the spleen, most DCs are lymphoid-tissue-resident, while tissues contain migratory DCs, that may derive from monocytes (107, 266). When the phenotypes of $CD11c^+$ cells from spleen and peritoneal cavity were compared by flow cytometery, we noted several differences. In the peritoneal cavity only a minority of the cells expressed high levels of CD11c while the major population expressed intermediate levels of CD11c in combination with CD11b. (Appendix I, Fig. 1). Both the $CD11c^{int}CD11b^+$ cells and the $CD11c^{high}$ cells expressed high levels of MHC II, but only the $CD11c^{int}CD11b^+$ cells expressed some F4/80, while the $CD11c^{hi}$ cells were negative for this marker. We speculate that the $CD11c^{int}CD11b^+$ cells represent a monocyte-derived type of DC.

Though the majority, 2/3, of the $CD11c^+B220^-$ cells in the spleen expressed high levels of CD11c, the rest, 1/3, had a $CD11c^{int}CD11b^+$ phenotype (Appendix I, Fig. 1). These cells are likely to represent a type of monocyte and we speculated that they could be of similar origin and have similar functions as the peritoneal $CD11c^{int}CD11b^+$ cells. However, the expression of MHC II on the splenic

$CD11c^{int}CD11b^{+}$ cells was not uniformly high as in the peritoneal cavity, but was very variable.

As a comparison, we analysed the $CD11c^{+}B220^{-}$ cells in the Peyer´s patches (Appendix I, Fig. 1). The frequency of $CD11c^{+}B220^{-}$ cells in the Peyer´s patches was almost ten times lower, than in the spleen and peritoneal cavity and they constituted only around 0.15% of the total number of cells. The vast majority of the $CD11c^{+}B220^{-}$ cells in the Peyer´s patches expressed high levels of CD11c and only a few percent had the phenotype of $CD11c^{int}CD11b^{+}$ cells.

APCs from the Peyer´s patches are inefficient in presenting soluble antigens

Next, we compared the abilities of unfractionated and CD11c-enriched cells from Peyer´s patch, spleen and the peritoneal cavity to induce OVA-specific T cell activation to *E. coli*-OVA and various doses of soluble OVA. In general, DCs from the peritoneal cavity induced the highest T cell proliferation, followed by splenic APCs, while the Peyer´s patch APCs were least efficient (Appendix 1, Fig. 2 A). This can probably at least partly be explained by the lower frequency of $CD11c^{+}B220^{-}$ cells in the Peyer´s patches. We noted that the differences in antigen-presenting capacity by APCs from the different compartments, was greatest for presentation of low doses of soluble OVA (Appendix 1, Fig. 2 B). Using either 10 or 100 µg/ml dose of OVA, peritoneal APCs induced significantly higher T cell proliferation than APCs from the spleen or Peyer´s patches. At the 100 µg/ml OVA dose, splenic APCs induced a significantly higher T cell proliferation than Peyer´s patch APCs, $p < 0.05$, according to Student's t-test.

$CD11c^{hi}$ and $CD11c^{int}CD11b^{+}$ cells can both present antigens to $CD4^{+}$ T cells

Next, we compared the abilities of $CD11c^{hi}$ and $CD11c^{int}CD11b^{+}$ cells to present bacterial and soluble antigens to $CD4^{+}$ T cells. $CD11c^{+}B220^{-}$ cells from the Peyer´s patches and $CD11c^{high}B220^{-}$ and $CD11c^{int}B220^{-}CD11b^{+}$ cells from the spleen were sorted by flow cytometry. Purified APCs (5,000 cells/well) were pulsed with *E. coli*-OVA, *E. coli*-control or soluble OVA, washed and co-cultured with $CD4^{+}$ T cells. The purified $CD11c^{hi}$ cells from the Peyer´s patches and the spleen induced T cell proliferation to *E. coli*-OVA as well as high doses of soluble OVA (500 µg/ml), though we did not detect T cell proliferation to a lower dose of OVA (100 µg/ml) using APCs from the Peyer´s patches (Appendix 1, Fig. 2C). The $CD11c^{int}CD11b^{+}$ cells from the spleen presented bacterial and soluble OVA (500 µg/ml) with similar efficiency as did the splenic $CD11c^{hi}$ cells (Appendix 1, Fig. 2C). We have not yet tested the ability of the $CD11c^{int}CD11b^{+}$ cells to present lower doses of OVA.

$CD11c^{hi}$ and $CD11c^{int}CD11b^{+}$ cells can both phagocytose bacteria

As endocytosis/phagocytosis is a prerequisite for presentation to T cells, we tested the capacity of the $CD11c^{+}B220^{-}$ cells in various tissues to phagocytose GFP-expressing or pHrodo stained bacteria, using flow cytometry. Both the $CD11c^{hi}$ and $CD11c^{int}CD11b^{+}$ cells phagocytosed bacteria, but the $CD11c^{int}CD11b^{+}$ cells more frequently contained fluorescent bacteria. The $CD11c^{int}CD11b^{+}$ cells in the spleen and peritoneal cavity were at least twice as effective as the $CD11c^{hi}$ DCs in taking up *E. coli* (data not shown). We tried also to study uptake of FITC-stained OVA, but the data were inconclusive, since it was difficult to differentiate between surface bound and internalised OVA. Further experiments are needed to reveal if the $CD11c^{int}CD11b^{+}$ cell population in the spleen really is able to present antigens and if it in that case is better than the $CD11c^{hi}$ population at presenting low doses of soluble antigen.

GENERAL DISCUSSION

Gram-positive and Gram-negative bacteria and bacterial components have various effects on innate and adaptive immune responses. The effects are generally activating, alerting the immune system of danger, but several mechanism, innate as well as adaptive, also exist that dampen excessive immune responses.

In human monocytes, Gram-positive bacteria induce much higher production of IL-12 and slightly higher levels of TNF, than Gram-negative bacteria (103, 128). In contrast, Gram-negative bacteria are more potent inducers of IL-10 and PGE_2, than are Gram-positive bacteria (103, 127). IL-12 induces production of IFN-γ by NK and T cells. A key function for TNF and IFN-γ in the immune defence is to, via enhancing phagosome maturation, increase the ability of the phagocyte to kill its prey. As Gram-positive bacteria have a thick and sturdy cell wall, it may be of physiological importance for monocytes to produce high levels of phagocyte activating cytokines in response to these bacteria.

We observed that an intact cell wall was an absolute requirement for Gram-positive bacteria to be able to induce IL-12 production in monocytes. Sonicated Gram-positive bacteria induced some TNF, but the levels were much lower than the levels induced by intact bacteria. Further, isolated components of the Gram-positive cell wall, such as LTA and peptidoglycan, can induce TNF in monocytes, but only when added in very high doses (128). In contrast, intact and sonicated Gram-positive bacteria induced similar levels of IL-6.

While there was a large difference between the IL-12 and TNF induced in response to intact and decomposed Gram-positive bacteria, the Gram-negative bacterium *E. coli* induced similar levels of all cytokines tested including IL-12, TNF, IL-6, IL-8 and IL-10 regardless of if it was intact or sonicated. Further, isolated LPS has been shown to be of similar potency as intact *E. coli* in inducing these cytokines (128). Thus, while intact as well as decomposed *E. coli* appears to signal mainly via its LPS component, intact and decomposed Gram-positive bacteria appear to utilise different signalling pathways, with only the intact bacteria triggering IL-12 production and strong TNF production.

Using bifidobacteria as a model organism, we tried to elucidate the molecular background to the strong ability of Gram-positive bacteria to induce IL-12 production in human monocytes. We observed that phagocytosis was crucial for production of IL-12 by monocytes in response to bifidobacteria, as cytochalasin treatment completely prevented this production. IL-6, which was measured as a comparison, was only moderately affected by the cytochalasin treatment. After phagocytosis, vesicles from the Golgi network are recruited in a process called

phagosome maturation. Substances needed to kill and decompose bacteria are delivered, but also pattern recognition receptors, such as TLR2 and TLR4. The enzyme PI3K is crucial for phagocytosis as well as phagosome maturation (88-90), and inhibiting PI3K with wortmannin strongly and dose-dependently inhibited IL-12 production in response to intact Gram-positive bacteria. Thus, phagocytosis and possibly phagosome maturation appeared to be required for induction of IL-12 in response to Gram-positive bacteria. The longer the bacteria reside within the phagosome, the more IL-12 is likely to be induced, as has been shown for lactobacilli, where the resistance to lysis within macrophages correlated to their abilities to induce IL-12 (316). We propose that the relevant pathway for IL-12 production is induced by receptor cross linking by intact Gram-positive bacteria from within the phagosome. Candidate monocytic receptors that may be triggered by Gram-positive bacteria in the phagosome include NOD2 and TLR2. The involvement of NOD2 may be of particular relevance, since lactobacilli or bifidobacteria stimulation of monocyte derived DCs from NOD2 -/- mice elicited lower production of IL-12 and TNF, but similar levels of IL-6 and IL-10, compared to wild type mice (105). Signalling pathways identified as important for IL-12 production in response to intact Gram-positive bacteria include JNK and NF-κB.

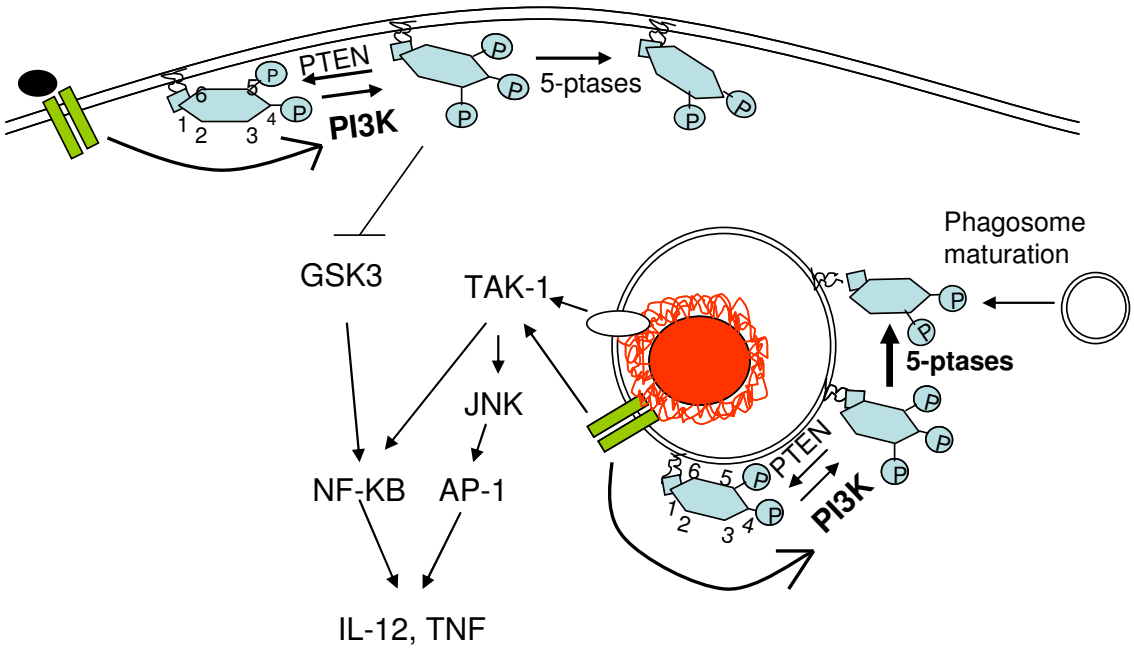

Figure. 9. Model for induction of IL-12 in response to Gram-positive bacteria, and inhibition of bacterial components. Phagocytosed bacteria signal from within the phagosome via receptors such as NOD2 and TLR2 to activate JNK and NF-κB. Bacterial fragments signal via surface TLRs, which may induce pathways that negatively regulate IL-12 and TNF production. Such a pathway could involve PI3K and inactivation of the constitutively active GSK3, which then is prevented from acting as a co-activator of NF-κB activation.

Decomposed bacteria should recognise the same receptors as intact bacteria. However, soluble ligands for NOD2 or TLR2 were not only inefficient inducers of IL-12, but their presence also inhibited IL-12 production in response to intact Gram-positive bacteria. How can it be possible for intact and fragmented bacteria to induce different signalling pathways? We propose that this is due to the fact that bacteria, but not soluble components, can be phagocytosed. IL-12 to Gram-positive bacteria might be triggered from within the phagosome. Thus, receptor activation at the monocyte surface, or possibly from within pinocytic vesicles, might not trigger the relevant pathway for IL-12 production, but instead generate a negative signal (Fig 9). The ability of bacterial fragments to turn off the pathways triggering phagocyte activation, could be of physiological relevance. Once the macrophage has achieved its goal, to kill and decompose the microbe, no further activation is needed.

S. pneumoniae is a major human pathogen. The thick pneumococcal capsule minimizes phagocytosis and is the most important virulence factor. Another virulence trait is its tendency to undergo autolysis in stationary phase, through activation of the cell wall bound enzyme LytA that breaks down peptidoglycan. The function of autolysis in pneumococcal pathogenesis is unclear, but must relate to the release of cytoplasmic and cell wall components upon autolysis of pneumococci with reduced virulence. As we had noted that intact Gram-positive bacteria were potent inducers of IL-12, regardless of if they were dead or alive, but that fragments of Gram-positive bacteria could counteract IL-12 production, we speculated that pneumococci, with the ability to undergo autolysis, would be poor inducers of IL-12 in monocytes. Indeed, pneumococci grown to stationary phase induced undetectable levels of IL-12, and compared to related *Streptococcus* strains of the *viridans* group, very low levels of TNF and IFN-γ in PBMC. However, the other cytokines measured, i.e. IL-6, IL-8 and IL-10, were induced in similar levels by pneumococci and *Streptococcus viridans* strains. When autolysis was prevented, pneumococci induced significantly higher levels of IL-12, TNF and IFN-γ in PBMC. However, compared to the *Streptococcus viridans* strains, the intact pneumococci were still poor inducers of IL-12 and TNF. As we had seen that phagocytosis was crucial for IL-12 production, we speculated that the capsule, hindering phagocytosis, was an additional factor impeding induction of phagocyte activating cytokines. Indeed, a non encapsulated variant of *S. pneumoniae* did elicit high levels of IL-12 as well as TNF. We concluded that two of the pneumococcal virulence factors selectively prevented production of phagocyte activating cytokines by PBMC. The capsule prevented the pneumococci from triggering of high levels of IL-12 and TNF, presumably since it minimizes phagocytosis. Autolysis of bacteria with reduced viability generated fragments which selectively turned off production of phagocyte activating cytokines, presumably by taking advantage of a natural feed back system to signalling to monocytes that the microbe is defeated. The fragments generated by autolysis of *S. pneumoniae* also inhibited

phagocytosis and intracellular killing of viable pneumococci, presumably by sterical hindrance.

When pneumococci decompose, in addition to cell wall fragments, also a toxin called pneumolysin is released. Pneumolysin lacks a secretion signal, it is therefore mainly released when the bacteria undergo autolysins (171). Though it is still controversial, pneumolysin may also be released by an undefined LytA-independent mechanisms, at least in certain strains (194). However, the bulk of pneumolysin is probably released by the action of autolysins.

Pneumolysin is a multifunctional cytolysin, which in high concentrations is toxic to most cell types. Aside the capsule, pneumolysin is regarded as the most important pneumococcal virulence factor. Though pneumolysin can activate complement and stimulate TLR4, most of its actions occur via haemolytic interactions with host cell membranes. Mutant variants of pneumolysin exist that lack hemolytic activity, which makes it possible to investigate which effects on the host that are dependent on the haemolytic activity of the toxin, and which are not. When present in high doses haemolytic active pneumolysin forms large pores and lyses the host cells. Also in lower doses, not related to formation of proper pores, the haemolytic active pneumolysin, but not mutant pneumolysin, induces apoptosis in neurons and inhibits ciliary beating (160).

We discovered a novel mechanism by which haemolytic active pneumolysin interacts with host cells. Only when human neutrophils were stimulated with autolysed, but not intact pneumococci, they produced oxygen radicals. We identified pneumolysin, which was released by the action of autolysins, as the ROS-inducing agent. Only haemolytically active pneumolysin induced ROS-production, and the induction was totally dependent on the influx of extracellular Ca^{2+}, that occurred upon stimulation of neutrophils with native pneumolysin. Further, the radicals formed were mainly confined to an intracellular compartment.

Intracellular ROS was clearly formed into compartments devoid of bacteria. We can think of at least three possible functional consequences of the high intracellular oxygen production into empty intracellular stores. Firstly, the normal reaction pattern of neutrophils may change. This was indeed observed to occur, since priming with haemolytic active, but not mutant, pneumolysin resulted in a ROS response to the bacterial ligand fMLF, which was sustained over a much longer period of time, than in normal, non-primed, cells. This may imply that the neutrophil cytoskeleton function was disturbed, since an active cytoskeleton is known to be involved in terminating the response to fMLF.

Secondly, we speculate that the neutrophils may be more likely to die after their pneumolysin induced oxidative burst. That would be beneficial for the pneumococci, since their worst enemy then would be defeated, without having

phagocytosed bacteria. Pneumolysin induces cell death through pore-formation in most cells when present in high doses (around 1µg/ml), and has also been shown to induce apoptosis in several cell types at lower doses. We observed oxygen radical production in low doses (down to 2 ng/ml), normally not related to cell death. We did look at necrosis and apoptosis in neutrophils in response to various doses of haemolytic active and mutant pneumolysin, but only after 30 min of stimulation. At this early time point we could not observe any differences in apoptosis induced by native and mutant pneumolysin, but the native pneumolysin induced more necrosis than the mutant variant. This was seen in particular when using high doses, but also to some extent with lower doses. It is possible that we would have observed more cell death at a later time point after stimulation. Pneumolysin induced a Ca^{2+} flux in neutrophils, which in most cell types is related to increased apoptosis, but in neutrophils a Ca^{2+} flux has been shown to inhibit apoptosis (317). Thus if the Ca^{2+} flux and/or oxygen radical production would render the neutrophils sensitive to dying, necrosis may be a more likely cause than apoptosis.

A third possible consequence of the high ROS-production in neutrophils upon pneumolysin stimulation could be that the neutrophils are induced to form neutrophil extracellular traps (NETs). NETs are used to capture and kill extracellular bacteria, and is a way for the neutrophils to participate in host defence beyond their lifespan. In neutrophils, oxygen radical production via the NADPH-oxidase is a prerequisite for the formation of NETs. Neutrophils have been demonstrated to form NETs in response to *S. pneumoniae* (318), but whether or not NET-formation is induced by purified pneumolysin is not known.

In addition to studying innate mechanisms activated in response to bacteria and bacterial products, we also examined adaptive $CD4^+$ T cell responses to an antigen presented as a bacterial protein either inside Gram-positive or Gram-negative bacteria. The route by which the antigen enters the body, the type of antigen-presenting cell and the physical form of the antigen are factors involved in determining the magnitude and nature of the $CD4^+$ T cell response. Using natural populations of antigen presenting cells from the spleen or the peritoneal cavity, we compared the $CD4^+$ T cell responses to the same antigen in its soluble form or when expressed within a Gram-positive or Gram-negative bacterium.

In initial experiments we compared the effectiveness of soluble OVA and OVA expressed within *E. coli*, to induce proliferation of OVA-specific $CD4^+$ T cells, isolated from the OVA transgenic DO11.10 mouse. We noted that OVA was much more immunogenic when present in the context of *E. coli*, as compared to when present in its soluble form. At least a 500 times higher dose of soluble, than "bacterial", OVA was needed for an OVA-specific proliferation of similar magnitude, using unfractionated splenocytes as APCs. We compared the production of typical T cell cytokines in the co-cultures after 4 days and noted that when APCs had been pulsed with soluble OVA, large amounts of IL-13 was

produced in the co-cultures, while when OVA was presented in the context of *E. coli*, both OVA-specific IL-13 and IFN-γ was produced in the co-cultures. In addition, large amounts of IL-10 was produced in the *E. coli* containing cultures, but the majority of this IL-10 was not produced by the OVA-specific T cells, but probably macrophages, or other cells present in the culture.

Next we wanted to study the T cell responses induced when OVA was expressed within a Gram-positive bacterium. Our constructs supporting OVA-production in *E. coli* were not functional in the Gram-positive strains tested. We therefore constructed new OVA-vectors, which were optimised to function in lactobacilli. To allow high protein expression in lactobacilli a synthetic gene, coding for a fragment of OVA but modified to comply with the codon-usage in lactobacilli, was constructed and inserted into expression vectors functioning in lactobacilli. The OVA-fragment of this construct was efficiently expressed by *Lactobacillus sakei* and *Lactobacillus plantarum.* Also *Lactococcus lactis* produced the OVA-fragment, but in lower concentration. To allow comparison with Gram-negative bacteria, the same OVA-fragment was also cloned into *E. coli.*

At first we used splenocytes to test if the OVA-fragment expressed in the lactobacilli/lactococci and *E. coli* was immunogenic. When expressed in *E. coli*, a potent OVA-specific T cell proliferation was induced by the OVA-plasmid. The magnitude of the T cell proliferation, as well as the types and amounts of cytokines produced in the co-culture, was very similar to the responses observed using *E. coli* expressing the entire OVA-protein. However, when OVA was expressed in the Gram-positive bacteria, the OVA-specific T cell proliferation induced was very poor. *Lactobacillus plantarum* NC8 is known to have a very thick and sturdy cell wall, difficult to digest with lyzosyme. When OVA was expressed in this bacterium, there was not even a tendency of OVA-specific T cell proliferation. When splenic APCs were pulsed with *Lactobacillus sakei* or *Lactococcus lactis* there was a tendency of OVA-specific T cell proliferation and using Student's paired, but not unpaired, t-test the proliferation induced in response to *L. sakei*-OVA was significantly higher than the proliferation induced to *L. sakei*-control. In accordance with the low magnitude of proliferation, only moderate levels of T cell cytokines were produced in the cultures. More IFN-γ and IL-17 was produced in lactobacilli stimulated cultures when the DO11.10 T cells were added, than without T cells. Thus, thus these cytokines were not likely to be produced by APCs. A weak tendency to OVA-specific IFN-γ was noted, but there was no significant difference between the production of these cytokines in response to *L. sakei*-OVA and *L. sakei*-control.

As the Gram-positive bacteria appeared to be inefficiently presented by splenic APCs, we tested the ability of APCs from the peritoneal cavity to present OVA expressed in lactobacilli. $CD11c^+$ peritoneal cells were efficient presenters of *E. coli*-OVA and soluble OVA to $CD4^+$ T cells. They could also present *L. sakei*-OVA

in a manner resulting in significant OVA-specific T cell proliferation. However, similar to when splenocytes were used to present the antigen, the magnitude of the $CD4^+$ T cell response to *E. coli*-OVA was much higher than to *L. sakei*-OVA.

We did not observe significant OVA-specific cytokine production in response to OVA expressing *L. sakei*, though there was a tendency of higher IL-10 production in response to *L. sakei*-OVA, than *L. sakei*-control. Similar to when splenic APCs were pulsed with lactobacilli, a T cell dependent, but non-OVA-specific, production of IFN-γ and IL-17 was observed. We speculated that cells such as NKT cells may have been present among the DO11.10 cell added to the APCs, since although the purified DO11.10 cells all bear the Tg TCR, some of them do not express CD4. They might produce these cytokines in response to factors, such as IL-12, IL-18 or IL-1β, produced by lactobacilli stimulated APCs.

Using peritoneal DCs to present *E. coli*-OVA, significant OVA-specific production of IL-17 and IL-10 was induced, which was not the case when splenic APCs were used as presenters. When peritoneal APCs were used to present soluble OVA, the T cells proliferated vigorously, but only low levels of the measured cytokines (IFN-γ, IL-13, IL-17, IL-10) were produced. It is possible that the T cells were producing other cytokines, such as IL-9, which we did not look for.

A switch from an OVA-specific production dominated by IFN-γ and IL-13 towards more OVA-specific IL-17 and IL-10 was the overall pattern noted when peritoneal APCs were used instead of splenic APCs. We wondered which factors that mediated this switch. We could think of two possible explanations, not mutually exclusive. Firstly, mediators produced by APCs and bystander cells in the spleen and peritoneal cavity may differ. Secondly, the APCs at these sites might belong to different subsets intrinsically favouring different types of immune reactions.

Indeed different cytokines were produced by spleen and peritoneal cells when stimulated with bacteria. Splenocytes produced more IL-12 than the peritoneal cells, especially when stimulated by lactobacilli. Since soluble OVA mainly induced a IL-13 response in the spleen co-cultures, this might be the default polarisation for non-activated splenic APCs. The presence of IL-12 may be one of the factors skewing T cell responses induced by splenocytes towards more IFN-γ production. *E. coli* were not efficient inducers of IL-12 and, in accordance, the proposed skewing towards IFN-γ was only partly effective, as *E. coli*-OVA also induced high amounts of IL-13. Lactobacilli produced more IL-12 in the spleen cultures, and upon addition of DO11.10 T cells, a tendency to OVA-specific IFN-γ, but not IL-13 was noted.

When peritoneal cells were stimulated with bacteria, enormous amounts of PGE_2 and IL-6 and high levels of IL-10 was produced. Further, peritoneal macrophages have been shown to produce TGF-β (266), though we did not measure the levels of

this cytokine. The combination of IL-6, TGF-β and PGE_2 has in *in vitro* cultures been shown to be able to promote polarisation of T cell towards the Th17 linage (276, 289, 301). Thus, in this environment it may seem logical with an increased polarisation towards IL-17 producing cells. IL-10 can be co-produced by T cells polarised in various directions, including Th1, Th2, Th17 and regulatory T cells (289-291), so it is difficult to speculate about the reason for the increased OVA-specific production of IL-10 than was noted using peritoneal APCs. However, higher levels of IL-10 were produced by the peritoneal, than spleen, cells, in response to both *E. coli* and *L. sakei*, which may be one factor favouring IL-10 producing T cells.

Another explanation to the observed differences in cytokine production by T cells using splenic and peritoneal APCs, could be that the antigen-presenting cells in the two compartments differ. In both compartments, the $CD11c^+$ cells were identified as the major antigen presenters. However, when comparing the phenotypes of the $CD11c^+$ cells in the spleen and peritoneal cavity, several differences were noted. In steady state, the DC population in the spleen consists of mainly lymphoid-tissue resident DCs that originate directly from bone marrow precursors and not via monocyte intermediary (106, 107). In contrast, the migratory DCs in the tissues can be derived from monocytes (106, 107). The various subpopulations of $CD11c^{high}$ DCs in the spleen have been extensively studied (Table 4), but the DC populations of the peritoneal cavity are less well defined. When the peritoneal cells were stained with various DC, monocyte and macrophage markers and analysed by FACS, we noted that they appeared to closely resemble the previously described $CD11c^+$ cells in the lamina propria (319) (Table 4). The largest $CD11c^+$MHC II^+ population in the peritoneal cavity expressed intermediate levels of CD11c in combination with high levels of CD11b and low levels of F4/80.

In the spleen a reasonably large population of cells had a similar $CD11c^{int}CD11b^+$ phenotype as the dominating DC type in the peritoneal cavity (Table 4). These cells were highly phagocytic, expressed variable levels of MHC II, and were after cell sorting able to present both soluble and bacterial OVA to $CD4^+$ T cells. The splenic steady state $CD11c^{int}CD11b^+$ population has in the literature been described as $CD11c^{int}$ cells originating from monocytes, in contrast to the $CD11c^{hi}$ DCs, that originate directly from bone marrow precursors (106, 107). Cells with this phenotype have also been reported to accumulate in the spleen upon infection with *Listeria* and *Salmonella* (247, 248, 320). When recruited during bacterial infections, the $CD11c^{int}CD11b^+$ cells do not appear to participate in antigen-presentation, though they are efficient bacterial killers. As these cells during inflammation have been shown to produce NO, this may be a factor preventing efficient T cell priming.

DC/MØ population	Origin	Special function
Spleen		
$CD11c^+CD8\alpha^+CD103^+$	BMP	Cross-presentation, Th1 induction (230, 236)
$CD11c^+CD11b^+$	BMP	Th2 induction (230)
$CD11c^+CD11b^+CD4^+$	BMP	Broadly the same as $CD4^-$ DC
$CD11c^{int}CD11b^+$	monocyte	?
Peyer´s patches		
$CD11c^+CD8\alpha^+$	Probably BMP	Produce IL-12 in response to microbes, induce Th1 (321)
$CD11c^+CD11b^+$	?	Produce IL-10 and IL-6, induce Th2, IgA (321-323)
CD11c+ $CD8\alpha^-CD11b^-$	?	Produce IL-12 in response to microbes, induce Th1 (321)
Lamina propria		
$CD11c^+CD103^+$	?	Migrate to MLN during steady state, present soluble antigens, induce gut-homing markers, efficient cross-presentation (238)
$CD11c^+CD11b^+CX3CR1^+$	?	Induce Th17 polarisation (319). Sample bacteria from intestinal lumen via dendrites? (324).
$CD11c^{int}CD11b^+CX3CR1^+$ $F4/80^{lo}$	monocyte	Spontaneous IL-10 production, Induce Treg in the presence of TGF-β (319). Sample bacteria from intestinal lumen via dendrites? (324).
Lungs		
$CD11c^+CD103^+$	monocyte	Migrate to LN during steady state, efficient cross-presentation (241).
$CD11c^+CD11b^+CX3CR1^+$	monocyte	Migrate to LN during steady state, efficient $CD4^+$ T cell activation (241).
$CD11b^+F4/80^{hi}CD11c^+SSC^{hi}$	monocyte	Inhibits lymphocyte proliferation
Peritoneal cavity		
$CD11c^+CD11b^-$ cells	?	?
$CD11c^+CD11b^+$	?	?
$CD11c^{int}CD11b^+F4/80^{lo}$	?	?
$CD11b^+F4/80^{hi}CD11c^-SSC^{hi}$	monocyte	Inhibits lymphocyte proliferation

Table 4. DC and macrophage populations present in various compartments during steady state. The DCs in the peritoneal cavity may be of similar phenotype as in the lamina propria. The $CD11c^{int}CD11b^+$ population in the spleen may be similar to the tissue DCs. Except for in the CD11c expression, the peritoneal macrophages are phenotypically similar to lung macrophages. BMP = bone marrow precursor

As far as I know, the ability of the $CD11c^{int}CD11b^{+}$ cells, present in the spleen during steady state, to act as antigen-presenters has not previously been addressed. We have only sorted these cells once and can not exclude that a low number of $CD11c^{hi}$ DCs may have been contaminating the population, but our working hypothesis is that these cells represent a monocyte derived splenic DC population, which during steady state participate in antigen-presentation to T cells.

We have also compared the antigen-presenting capacity of APCs isolated from the Peyer´s patches, spleen and peritoneal cavity. In the Peyer´s patches, the conventional DCs are of lower frequency than in the spleen and peritoneal cavity, and $CD11c^{int}CD11b^{+}$ cells are virtually undetectable. In general, the APCs from the peritoneal cavity induced the highest T cell proliferation, followed by splenic APCs, while PP APCs were least efficient in inducing T cell proliferation. The same pattern was found for presentation of soluble OVA and *E. coli*-OVA, but the differences were most pronounced for low doses of soluble OVA. Though the observed differences may have many reasons, we speculate that the different frequency of $CD11c^{int}CD11b^{+}$ cells in the different compartments could be a possible explanation. The $CD11c^{int}CD11b^{+}$ cells were most frequent among the peritoneal cells, followed by the spleen cells, while they were few in the PP. Their high capacity to take up antigen, may render them efficient presenters of especially low doses of soluble antigen.

An additional observation we made during our experiments with APCs from the peritoneal cavity, was that the antigen-presenting capacity of adherent peritoneal cells was confined to the $CD11c^{+}$ DC population and not to the $CD11c^{-}$ macrophage population. Only the $CD11c^{+}$ cells expressed high levels of MHC II, and the FSC, SSC pattern of the two populations was strikingly different. Further, the $CD11c^{+}CD11b^{+}$ cells expressed low levels of F4/80, while most of the $CD11c^{-}$ $CD11b^{+}$ cells expressed high levels of F4/80. I therefore think it is reasonable to define the $CD11c^{-}CD11b^{hi}F4/80^{hi}$ cells as resident peritoneal macrophages, while the $CD11c^{+}CD11b^{+}$ may represent monocyte derived tissue DCs.

The $CD11c^{+}$ peritoneal DCs presented *L. sakei*-OVA, *E. coli*-OVA and soluble OVA to $CD4^{+}$ T cells. The addition of $CD11c^{-}$ peritoneal macrophages to the DC-T cell co-cultures strongly inhibited T cell activation in response to *E. coli*-OVA. However, the presence of peritoneal macrophages only marginally affected T cell responses induced in response to soluble OVA. This implies that factors produced in response to bacteria stimulation were causing the inhibition. Peritoneal macrophages are phenotypically similar to alveolar macrophages (Table 4). Both peritoneal and alveolar macrophages have been shown to inhibit lymphocyte proliferation in spleen cultures activated with polyclonal stimuli (262, 264, 265). The inhibitory mechanisms have in these settings mainly been attributed to NO produced by macrophages, which via a direct effect on the cGMP levels in the lymphocytes reversibly inhibited their proliferation (325). However, the ability of

peritoneal macrophages to inhibit responses to bacteria has, to the best of my knowledge, not been demonstrated previously. Instead, addition of inflammatory cytokines to the macrophages in the polyclonal stimuli models has been shown to dampen their inhibitory effects on lymphocyte proliferation (265, 326).

A major inhibitory effect of T cell proliferation, was in our system only seen when the macrophages were activated with bacteria. We therefore suspected that bacteria stimulated macrophages produced inhibitory factors, mediating the effect. Indeed, soluble factors produced in response to bacteria stimulation, strongly inhibited T cell priming in antigen pulsed DC-T cell co-cultures. Several macrophage derived factors may contribute to the inhibition, including PGE_2, IL-10 and TGF-β. However, the contribution of NO appeared to be minor in this system. Interestingly, we also noted that the various factors produced by macrophages may influence the T cell polarisation in various ways. TGF-β was the only factor indentified as being a major inhibitor of IL-13 production, and NO was the only factor with major effects on the OVA-specific IL-17 production. We also noted that NO was critically important for the non-OVA-specific production of IFN-γ and IL-17 in response to *E. coli*-control. Thus, the data imply that NO, produced in response to *E. coli*, may activate cells such as NKT cells (or NK cells or T cells) to produced IFN-γ and IL-17 in the absence of antigen-specific activation.

To summarise the cytokine production in the co-cultures; the OVA-specific $CD4^+$ T cells could produce IFN-γ, IL-13, IL-17 and IL-10, and the relative amount depended both on the context of the antigen and the source of APC. The APCs produced IL-10 in response to bacteria stimulation, and IFN-γ and IL-17 was produced in a non-OVA specific manner by the DO11.10 cells. We think NKT cells could be present in the purified DO11.10 population and responsible for this production, but further experiments are needed to test this hypothesis. Of note, IL-13 was never produced in a non-antigen specific manner, neither to *L. sakei* nor to *E. coli*.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Bakterier kan delas in i två huvudgrupper baserat på hur deras cellvägg är uppbyggd. Gram-positiva bakterier har robusta cellväggar, bestående av ett tjockt lager med peptidoglykan, medan Gram-negativa bakterier har en tunnare cellvägg som även innehåller LPS, en molekyl som inducerar kraftig inflammation.

En viktig immuncell i det tidiga försvaret mot bakterier är monocyten/makrofagen. Det är en vit blodkropp som kan äta upp och bryta ner bakterier som kommer in i kroppen. Makrofager som ätit upp bakterier producerar olika typer av signalmolekyler, bland annat cytokiner. En viktig funktion för cytokinerna är att locka andra immunceller till platsen. Neutrofilen är en av de celler som lockas till inflammationshärden av de cytokiner som makrofagen producerar. Liksom makrofager äter neutrofiler upp och dödar bakterier. Makrofager och neutrofiler kallas med ett gemensamt namn för fagocyter (fago=äta, cyt=cell). Cytokiner från makrofagerna kan också styra vilken typ av immunsvar kroppen ska använda sig av för att bekämpa bakterien, genom att aktivera olika typer av lymfocyter.

Vår grupp har tidigare upptäckt att monocyter/makrofager som stimuleras med Gram-positiva respektive Gram-negativa bakterier producerar olika typer av cytokiner. När monocyter träffar på Gram-positiva bakterier bildar de stora mängder av fagocyt-aktiverande cytokiner, vilka gör fagocyterna bättre på att bryta ner och avdöda bakterier. Detta kan vara viktigt, då det torde vara svårare att sönderdela den tjocka Gram-positiva cellväggen, än den tunna Gram-negativa cellväggen.

Vi har undersökt den molekylära bakgrunden till de Gram-positiva bakteriernas starka förmåga att få monocyterna att producera den fagocyt-stimulerande cytokinen IL-12. Det visade sig att Gram-positiva och Gram-negativa bakterier stimulerar IL-12 produktion via olika signalvägar inuti monocyten. Gram-positiva bakterier kunde bara inducera IL-12 om den först hade ätits upp av monocyter, men inte Gram-negativa bakterier. De Gram-positiva bakterierna verkar alltså ge upphov till IL-12-stimulernade signaler inifrån monocyten, medan de Gram-negativa bakterierna kan signalera bara genom att binda till monocytens yta. Signalvägarna som de Gram-positiva bakterierna använde sig av för att stimulera IL-12 produktion innefattade aktivering av PI3K och JNK. Tvärtom verkade aktivering av PI3K hindra Gram-negativa bakterier från att stimulera IL-12 produktion. Vi drog slutsatsen att Gram-positiva respektive Gram-negativa bakterier använder sig av fundamentalt olika signalvägar för att inducera produktion av IL-12 i monocyter. En annan intressant iakttagelse var att trasiga Gram-positiva bakterier inte fick monocyter att bilda IL-12, utan tvärt om stängde av denna produktion. Vi tror att

trasiga bakterier kan vara en signal att ”faran är över” och att fagocyterna ska sluta aktiveras.

I vår omgivning, på vår hud och på våra slemhinnor lever en stor mängd olika bakterier. En del har stor förmåga att orsaka sjukdom och kallas därför patogener, medan andra, så kallade normalflorabakterier, sällan orsakar sjukdom. Patogeniciteten är inte kopplad till cellväggen, då det finns både Gram-positiva och de Gram-negativa patogener. Den stora skillnaden är att patogenerna utvecklat strategier för att kunna invadera, sprida sig, skada och undgå kroppens immunförsvar. Anlag som ger bakterier dessa egenskaper kallas virulensfaktorer.

En patogen Gram-positiv bakterie som jag har fokuserat på i denna avhandling är pneumokocken. Pneumokocken är en av de bakterier som orsakar mest sjukdomar i världen och den kan ge lunginflammation, öroninflammation, hjärnhinneinflammation och blodförgiftning. Anledningen till att pneumokocker är så farliga är dess uppsjö av virulensfaktorer. Vi har fokuserat på två av dessa virulensfaktorer, nämligen autolysin och pneumolysin, och försökt förstå hur de bidrar till att öka pneumokockens sjukdomsframkallande förmåga.

Autolysin är en udda virulensfaktor. Det är ett enzym som sitter bundet i pneumokockens cellvägg och som spontant aktiveras då bakterien mår dåligt. Det aktiverade autolysinet klipper sönder bakterie-cellväggen så att pneumokocken spricker. Det kan vara svårt att förstå att det skulle vara en fördel för bakterier att gå sönder, men eftersom autolysinet bara aktiveras när bakterien mår dåligt så är det troligt är att dessa bakterier snart skulle dö även om de inte klipptes sönder av autolysinet. Vi har undersökt de bakteriefragment som bildas då pneumokocker går sönder och upptäckt att de har flera effekter som gör kroppens immunförsvar mot ännu levande bakterier mindre effektivt. Intakta döda bakterier däremot är stimulerande för immunförsvaret på samma sätt som levande bakterier. En funktion som vi upptäckte hos de trasiga pneumokockfragmenten var att de kunde hindra makrofager och neutrofiler från att äta upp och döda ännu levande bakterier. Dessutom kunde fragmenten stänga av de signalvägar som behövdes för att monocyterna skulle kunna bilda de fagocyt-aktiverande cytokinerna (t.ex. IL-12) när de träffade på intakta Gram-positiva pneumokocker. Följden blev således att fagocyternas förmåga att äta upp och avdöda ännu levande pneumokocker blev mycket sämre i närvaro av trasiga bakterie-fragment. Man kan alltså tänka sig att om några få bakterier ”offrar sig” och går sönder, så skyddar de sina släktingar mot fagocytförsvaret.

Förutom att hämmande fragment bildas när pneumokocker gör sönder, så frisätts också en annan virulensfaktor som normalt ligger gömd inuti bakterien. Denna virulensfaktor heter pneumolysin, och är mycket giftigt. Flera studier har visat att pneumolysin är en av huvudorsakerna till att pneumokockinfektioner ger så stora vävnadsskador. Förutom att pneumolysin i höga doser skadar nästan alla celler i

kroppen, så verkar det ha en mängd effekter på immunsystemet även i lägre doser. Neutrofiler, som äter upp och avdödar bakterier, anses vara den enskilt viktigaste komponenten i vårt försvar mot pneumokocker. Vi undersökte om pneumolysin hade någon direkt effekt på neutrofilerna. Då neutrofilerna stimulerades med pneumolysin producerade de höga nivåer av fria syreradikaler. Neutrofiler använder sig normalt av syreradikaler för att avdöda bakterier som de ätit upp, men pneumolysin inducerade syreradikalproduktion i frånvaro av bakterier. För mycket syreradikaler orsakar vävnadsskador, och felaktig syreradikalproduktionen kan störa neutrofilernas normala reaktionsmönster. Således kan pneumolysin stimulera neutrofiler till att bilda overksamma syreradikaler, som stör det normala pneumokock-försvaret och skadar vävnaderna.

Den första försvarslinjen mot invaderande bakterier baseras på att makrofager och neutrofiler äter upp och avdödar mikroberna. Om denna typ av breda bakterieförsvar inte lyckas fullt ut, kan hjälp behövas från en mer specifik del av immunförsvaret, nämligen det adaptiva försvaret. Till skillnad från makrofager och neutrofiler, som reagerar på alla slags bakterier, så består det adaptiva förvaret av miljontals olika celler som reagerar på unika strukturer, så kallade antigener. Varje typ av bakterie innehåller en mängd olika antigener som skiljer sig från antigenerna i andra typer av bakterier. Om en adaptiv immuncell träffar på just det unika bakterieantigen som den känner igen, blir den aktiverad och hjälper makrofagerna och neutrofilerna att försvara kroppen specifikt mot just denna bakterie. Försvaret mot denna bakterie blir då koncentrerat och effektivt.

En celltyp i det adaptiva immunsystemet som vi har fokuserat på är T-hjälparcellen (som vi hädanefter enbart kallar T-cell). Beroende på hur en T-cell blir aktiverade kan den styra immunsvaret, mot dess unika antigen, i olika riktningar. De kan t.ex. stimulera makrofager och neutrofiler till att effektivare döda mikrober som innehåller detta antigen, hjälpa till vid antikroppsproduktion mot bakterier innehållande detta antigen eller stänga av immunsvar mot detta antigen, troligtvis för att begränsa vävnadsförstörelse. För att en T-cell ska bli aktiverad måste den träffa på sitt unika antigen presenterat på ytan av en annan cell. Dendritceller är en speciell typ av fagocyt som äter upp bakterier och har som huvuduppgift att presentera de bakteriella antigenerna på ytan för T celler.

Vår frågeställning var om Gram-positiva och Gram-negativa bakterier ger upphov till olika typer av T-cellssvar. T.ex. skulle man kunna tänka sig att Gram-positiva bakterier stimulerar fagocytaktiverande T-celler, medan Gram-negativa bakterier i högre grad inducerar T-celler som stimulerar antikroppsbildning. Eftersom varje T-cell bara känner igen en unik struktur, satte vi upp ett specialsystem för att möjliggöra jämförelser mellan T-cellsvar mot Gram-positiva och Gram-negativa bakterier. Vi fick Gram-positiva och Gram-negativa bakterier att producera äggalbumin (OVA), genom att föra in en gen som kodade för detta protein i bakterierna. Vi studerade sedan hur T-celler, som manipulerats så att de alla känner

igen en del av OVA istället för unika strukturer, reagerade när de träffade på äggalbunin i vanlig form, respektive som ett protein inne i Gram-positiva eller Gram-negativa bakterier.

I provröret fick dendritiska celler äta upp de OVA-producerande Gram-positiva, respektive Gram-negativa, bakterierna, samt lösligt OVA, och presentera OVAt för T-cellerna. Därefter jämförde vi hur T-cellerna reagerade på OVA som befunnit sig inuti en Gram-positiv bakterie, Gram-negativ bakterie eller i löslig form. OVA var mycket mer stimulerande när det befann sig i en bakterie, än när det var i sin vanliga lösliga form. Dessutom blev T-cellerna mycket mer aktiverade då de stimulerades med de Gram-negativa än de Gram-positiva bakterierna. Vi fann också att dendritiska celler från olika delar av kroppen (bukhålan respektive mjälten) aktiverade T celler på delvis olika sätt.

Sammanfattningsvis inducerar Gram-positiva och Gram-negativa bakterier olika typer av immunsvar både hos fagocyter och adaptiva T-celler. Troligt är att immunsvar mot Gram-positiva, respektive Gram-negativa, bakterier under årmiljonernas lopp har anpassats för att utgöra det mest effektiva skyddet mot respektive typ av bakterie. Dock har patogena bakterier, såsom pneumokocker, utvecklar strategier för att på ett eller annat sätt undkomma kroppens immunförsvar.

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