Inflammatory mediator response to Gram-positive and Gram-negative bacteria *in vitro* and in middle ear infections

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To my family

Inflammatory mediator response to Gram-positive and Gram-negative bacteria *in vitro* and in middle ear infections

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Abstract

Based on the structure of the cell wall, bacteria are divided into Gram-positive and Gram-negative. While the cell wall of Gram-positive bacteria is thick, the cell wall of Gram-negatives is very thin and surrounded by an outer membrane with LPS. Previous studies have shown that Gram-positive bacteria induce much more IL-12, TNF and IFN-γ from human peripheral mononuclear cells (PBMC) than do Gram-negatives, which instead induce more IL-6, IL-8 and IL-10 than do Gram-positive bacteria. In this thesis we confirm this cytokine pattern and show that the capacity of Gram-positive and Gram-negative bacteria to induce these distinct cytokine profiles is independent of their taxonomic relatedness. One exception from this general pattern was *Listeria monocytogenes*, which was a poor inducer of IL-12, TNF and IFN-γ despite being Gram-positive. Since an intricate interplay between IL-12, TNF and IFN-γ results in enhanced killing capacity of macrophages, these cytokines are recognized as phagocyte-activating cytokines. Another striking exception was *Streptococcus pneumoniae*, which induced no IL-12 despite its close relation to the viridans streptococci, which induced high levels. Aging pneumococci decompose due to the action of autolysin. We show that autolyzed pneumococci induce very little IL-12, TNF and IFN-γ and also inhibit the production of phagocyte-activating cytokines in response to intact bacteria. Further, fragments partly blocked phagocytosis of intact pneumococci. Thus, fragments generated by autolysin may paralyse phagocyte defenses and contribute to virulence.

To investigate the response to Gram-positive and Gram-negative bacterial infection, middle ear fluid was collected from children with acute otitis media (AOM) and from children with long-standing secretory otitis media (SOM). In SOM, Gram-negative bacteria were more prevalent than in AOM. Further, fluids with no cultureable bacteria were often positive by PCR in SOM, but not in AOM. This suggests that bacterial DNA is eliminated soon after killing of bacteria in AOM but not in SOM, or that bacteria remain dormant in the middle ear cavity of the SOM patients. The levels of inflammatory mediators in the fluids did not relate to the etiological agent. Instead, most cytokines, especially IL-1β, were highly elevated in middle ear fluids containing live, cultureable bacteria compared to negative fluids, even if microbial DNA could be detected by PCR. In contrast, high levels of IL-6 and PGE_2 were measured also in AOM fluids with no detectable bacteria, and might be important in the resolution phase of the infection.

Nasal spray treatment with viridans streptococci and lactobacilli were tested as a method to speed up resolution of SOM in a placebo controlled double blind pilot study. One third of children sprayed with viridans streptococci showed significant clinical improvement, while treatment with lactobacilli was less effective. Clinical recovery was not associated with changes in the nasopharyngeal flora, or the expression of inflammatory mediators in nasopharynx or in the middle ear. Spray treatment with viridans streptococci could be an alternative to surgery due to SOM, but the mechanism of the beneficial effects remains to be elucidated.

Key words: Gram-positive, Gram-negative, bacteria, cytokines, monocytes/macrophages, phagocytosis, *Streptococcus pneumoniae*, autolysin, acute otitis media, secretory otitis media, children, probiotics

LIST OF PUBLICATIONS

This thesis is based on the following studies, which will be referred to in the text by their Roman numerals (I-IV).

I. Skovbjerg S, Martner A, Hynsjö L, Hessle C, Olsen I, Dewhirst FE, Tham W, Wold AE. **Gram-positive and Gram-negative bacteria induced different cytokines from human mononuclear cells irrespective of taxonomic relatedness.** J Interferon Cytokine Res. 2010 Jan;30(1):23-32.

II. Martner A, Skovbjerg S, Paton JC, Wold AE. ***Streptococcus pneumoniae*** **autolysis prevents phagocytosis and production of phagocyte-activating cytokines.** Infect Immun. 2009 Sep;77(9):3826-37.

III. Skovbjerg S, Roos K, Nowrouzian F, Lindh M, Holm SE, Adlerberth I, Olofsson S, Wold AE. **High cytokine levels in perforated acute otitis media exsudates containing live bacteria.** Clin Microbiol Infect. 2009 Oct 14. [Epub ahead of print]

IV. Skovbjerg S, Roos K, Holm SE, Grahn Håkansson E, Nowrouzian F, Ivarsson M, Adlerberth I, Wold AE. **Spray bacteriotherapy decreases middle ear fluid in children with secretory otitis media.** Arch Dis Child. 2009 Feb;94(2):92-8.

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ABBREVIATIONS

AOM	Acute otitis media
ASC	Apoptosis-associated speck-like protein containing a caspase recruitment domain
CARD	caspase recruitment domain
ER	Endoplasmatic reticulum
IFN	Interferon
IL	Interleukin
LOS	Lipooligosaccharides
LPS	Lipopolysaccharides
LRR	Leucin-rich repeat
LTA	Lipoteichoic acid
MALT	Mucosa-associated lymphatic tissue
MAPK	Mitogen-activated protein kinase
MDP	Muramyl dipeptide
meso-DAP	*meso*-diaminopimelic acid
MHC	Major histocompatibility complex
NAD	NACHT-associated domain
NF-κB	Nuclear factor-κB
NO	Nitric oxid
NK	Natural killer
NLR	Nod-like receptor
NOD	Nucleotide-binding oligomerisation domain
PAMP	Pathogen-associated molecular pattern
PCA	Principal component analysis
PRR	Pattern recognition receptor
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PG	Prostaglandin
PYD	Pyrin domain
RSV	Respiratory syncytial virus
SOM	Secretory otitis media
TLR	Toll-like receptor
TNF	Tumour necrosis factor

INTRODUCTION

The human upper respiratory mucosa is colonized with a large number of bacteria. Whereas most bacteria are commensals and seldom cause disease in the immunocompetent host, some bacteria are potential pathogens. *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis* are all capable of invading the middle ear and cause acute otitis media (AOM), especially in children. Infection is associated with a strong inflammatory response against the invading bacteria. In this thesis we have studied the inflammatory mediator pattern elicited *in vitro* and *in vivo* in response to different bacteria in general and to otitis media pathogens in particular. We further investigated if this response could be modulated by treatment with probiotic bacteria in patients with long-standing middle ear fluid due to secretory otitis media.

Bacteria

After having discovered bacteria in the 1670th with a microscope of his own design, Antony van Leeuwenhoek wrote: "This was to me, among all the marvels that I have discovered in Nature, the most marvellous of all; and I must say for my part, that no greater pleasure has yet come to my eye than these spectacles of so many thousands of living creatures in a small drop of water moving among one another, each individual creature with its particular movement" (1).

It has been estimated that there are over 10^{30} individual bacteria worldwide, most of who are living in the ocean or in the soil (2). Bacteria have been found in extreme environments, like in the Greenland ice (3) and in acidic thermal springs (4), but numerous bacteria are also found in our own bodies.

Bacteria (prokaryotes) are unicellular organisms, about ten times smaller than mammalian cells. They lack many structures present in eukaryotic cells such as nucleus, Golgi apparatus and endoplasmatic reticulum. Their genetic material usually consists of a single chromosome located in the cytoplasm. Bacteria are often surrounded by a cell wall, a structure not found in mammalian cells. Structures of the bacterial cell wall are therefore potent activators of the innate immune system. Extensive remodelling and enlargement of the cell wall occur during growth and division. Some peptidoglycan degradation products are recycled, while much is shed, especially by Gram-positives, and possible to detect by the immune system. There are a few pathogenic bacteria that lack a cell wall, for example *Chlamydiae* and *Mycoplasma* spp. All other bacteria that possess a cell

wall are divided into two groups, namely the Gram-positive and the Gram-negative, respectively, based on the appearance of the cell wall.

Gram-staining

In 1884 the Danish scientist Hans Christian Gram published a staining method that is still widely used in the routine diagnostics as a first step for bacterial identification (5). After staining by crystal violet and fixation with iodine some bacteria remain blue after treatment with alcohol or acetone. These are called Gram-positive. Other bacteria are decolorized after alcohol treatment and appear red after counterstaining with safranin and are called Gram-negative. Some Gram-positive bacteria contain a fraction of cells that lose the blue dye easily and a culture of such bacteria appears as a mixture of Gram-positive and Gram-negative bacteria in the microscope. These are called Gram-variable.

The Gram-positive cell wall

A schematic illustration of the Gram-positive and Gram-negative cell wall is shown in Figure 1.

The Gram-positive cell wall consists of a rigid polymer, the peptidoglycan, which is up to 50 layers thick and surrounds the bacterium. The peptidoglycan is composed of chains of the alternating sugars *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM). Short peptide chains connect peptidoglycan strands to one another. In Gram-positive bacteria, the third amino acid in this peptide is usually L-lysine, while Gram-negatives instead generally have *meso*-diaminopimelic acid (*meso*-DAP) as the third amino acid residue (Figure 1). Exceptions to this rule are the Gram-positive bacteria *Listeria monocytogenes* and *Bacillus cereus*, which in common with Gram-negatives have *meso*-DAP instead of L-lysine in their peptide chain (6). Meso-DAP is recognized by the pattern recognition receptor NOD1 (page NOD127).

Most Gram-positive bacteria have teichoic acids and lipoteichoic acids in their cell wall, which are responsible for the negative charge of the bacteria, and, when present, are essential for the survival of the bacteria. Teichoic acids are anchored to the muramic acid of the peptidoglycan, and mostly built of long, repetitive units of glycerol or ribitol phosphates. Lipoteichoic acids, on the other hand, are long polymers of glycerol phosphate linked to a glycolipid anchored to the cell membrane (7, 8). Teichoic or lipoteichoic acids may be decorated with different residues, e.g. choline, which provides an anchor site for choline-binding proteins including pneumococcal autolysin (9). Choline is also antigenic and antibodies to phosphorylcholine are produced against many Gram-positive bacteria. Some Gram-positive bacteria also contain lipoproteins anchored to the cell membrane (10).

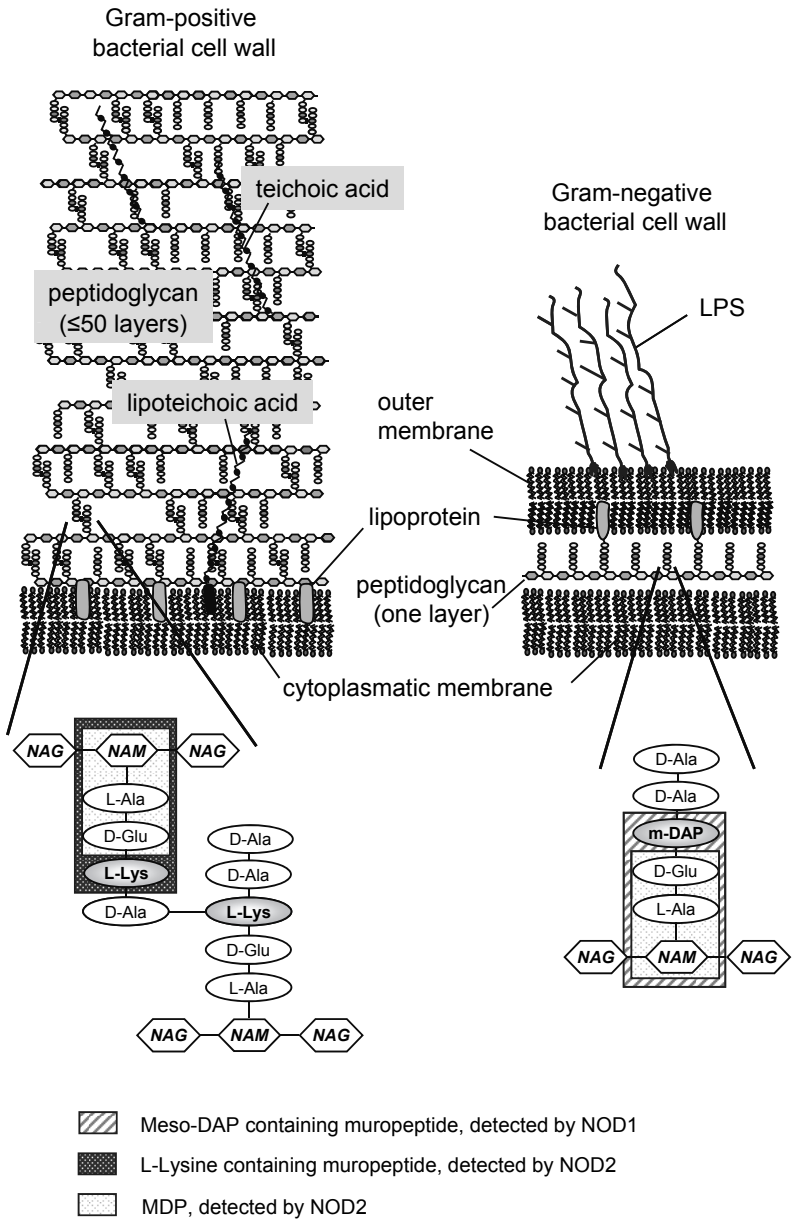

Figure 1: The composition of the Gram-positive and Gram-negative cell wall, respectively, modified from a figure by Agnes Wold. The short peptide chains connecting the Gram-positive peptidoglycan strands usually have L-lysine in the third position, while this residue is occupied by meso-DAP in Gram-negative bacteria.

The Gram-negative cell wall

In contrast to Gram-positive bacteria, the Gram-negatives have a thin cell wall, usually only consisting of one single layer of peptidoglycan. Anchored to the cell wall by lipoproteins is an outer membrane which contains lipopolysaccharides (LPS), the most potent danger signal known today. Minimal doses are enough to stimulate local as well as systemic inflammatory reactions. LPS is composed of the lipid A domain, the core and the O-antigen. Lipid A is a conserved structure composed of disaccharides linked to fatty acids that anchors LPS to the cell membrane. The exact function of lipid A in the bacteria is unknown, but it is vital for the survival of the bacteria, and is responsible for the strong immune activation by LPS (11). The core varies between bacterial genera and is composed of oligosaccharides. Farthest out is chains of repetitive trisaccharides sometimes provided with side chains, which vary between strains and constitute the immunogenic O-antigen. Some mucosal bacteria, including the genera *Haemophilus* and *Moraxella* lack the O-antigen and their LPS is usually referred to as lipooligosaccharides (LOS) (12). The LOS can vary considerably between and even within bacterial strains, and may be modified by host substances or by on-off switching of genes, a phenomenon called phase variation (13). These modifications are thought to enhance virulence and contribute to colonization and persistence in the host (14-16).

During Gram-staining cytoplasmatic proteins are stained by crystal violet. Fixation by iodine is thought to produce protein-dye complexes that are too large to pass through the tight meshwork of the Gram-positive cell wall. However, in Gram-negatives the dye-protein complexes leak out of the cell as the outer membrane is decomposed by alcohol, and the peptidoglycan layer is thin and looser due to few cross-links between chains. Hence, Gram-negative bacteria are decolorized.

Bacterial taxonomy

Carl von Linné, in his *Systema Naturae* (1735) introduced five categories (*species*, *genus*, *order*, *class* and kingdoms) for the systematic classifications of the natural world, including living species. The Linnaean system of taxonomy is still in use, complemented with the *family* and *phylum* categories, as well as *domain*, *superphylum*, *suborder, subclass* and *subspecies,* in certain cases. Historically, bacterial classification has been based mainly on morphology and biochemical tests. As techniques and approaches have improved, re-evaluation of bacterial taxonomy has been made continuously. Still, taxonomy is a dynamic process as modern molecular bacteriology gives new insight into the relationships of species. Examples of genotypic-based molecular techniques used currently include genomic DNA-DNA hybridization, sequencing of the 16S rRNA gene, or sequencing of multiple protein-coding genes (multi-locus sequence analysis; MLSA), to name a few. Along with the emergence of newer typing methods discussions of a proper species definition have been in progress. The recognized establishment of protocols

used for describing a bacterial species was proposed by Wayne *et al*. 1987 and defines strains with approximately 70% or greater DNA-DNA relatedness, with 5ºC or less difference in their melting temperature and with some differential phenotypic property (17). However, additional criteriae have been proposed as new methods are applied and the topic of bacterial systematics and the concept of bacterial species continues to be debated (18-20).

Streptococcus species

One example of species whose taxonomy has undergone extensive re-evaluation is the *Streptococcus* spp. Since long streptococci are divided into α-hemolytic or β-hemolytic streptococci based on their appearance on blood-containing agar plates. This approach is still used clinically to identify the more virulent β-hemolytic streptococci from species which usually are of low pathogenecity. Non-β-hemolytic streptococci are either α-hemolytic or non-hemolytic. Since expression of α-hemolysis depends very much on culture conditions, the term α-hemolytic is nowadays rather irrelevant. Another way of classifying streptococci is based on serological analysis of cell wall carbohydrates or teichoic acids according to the system developed by Lancefield. In this system, β-hemolytic streptococci are divided into the group A, C and G, but in non-β-hemolytic species, Lancefield typing is of limited value. In fact, classification of non-β-hemolytic streptococci is complicated, even sequencing of the 16S rRNA gene sometimes fails to distinguish between species, examplified by 99% similarity between *S. mitis*, *S. oralis* and *S. pneumoniae* (21). Therefore, both DNA-based methods and phenotypic characteristics are used for separation of species. Most non-β-hemolytic streptococci are referred to as viridans streptococci, which are divided into five groups, namely the *mutans*, *salivarius*, *anginosus*, *sanguinis* group and *mitis* groups (22). *S. pneumoniae*, often called pneumococcus, has due to genetic similarities been placed within the *Streptococcus mitis* group (23). Though genetically related, the pneumococci are far more virulent than the other streptococci in this group. Thus, taxonomic relatedness between species does not necessarily reflect their pathogenic capacity.

Phylum and Gram-staining pattern

It has been estimated that there are 10^7 to 10^9 bacterial species worldwide (24). There are at least 50 bacterial phyla, and half of them contain only bacteria that have never been cultured (24). Bacteria of medical interest are found in eight phyla, namely *Actinobacteria*, *Bacteroidetes*, *Chlamydiae*, *Firmicutes*, *Fusobacteria*, *Proteobacteria*, *Spirochaetes* and *Tenericutes*. The phyla *Bacteroidetes*, *Fusobacteria*, *Proteobacteria* and *Spirochaetes* contain Gram-negative bacteria only. The pathogenic species found in these phyla are generally rather easy to culture. An exception is the *Spirochaetes* phylum, whose members require other methods for diagnosis. The genera *Chlamydia* and *Mycoplasma* in the phyla *Chlamydiae* and *Tenericutes*, respectively, are also referred to as “Gram-negative”;

however, both genera entirely lack cell wall and *Mycoplasma* also lack cytoplasmatic membrane. The term "Gram-negative" is therefore rather irrelevant for these groups. Gram-positive bacteria with a high G+C content (e.g. *Bifidobacteria*, *Propionibacteria* and *Corynebacteria* spp.) reside in phylum *Actinobacteria*. Gram-positive bacteria with a low content of G+C (including staphylococci and streptococci, lactobacilli and C*lostridiae* spp.) are found in *Firmicutes*, but this phylum also contains the Gram-negative family *Veillonellaceae* with genera like *Centipeda*, *Megasphaera*, *Selenomonas* and *Veillonella*.

Bacteria in acute otitis media

The most common bacteria causing AOM are *S. pneumoniae*, *H. influenzae* and *M. catarrhalis*, whereas group A β-hemolytic streptococci (*Streptococcus pyogenes*) are uncommon today (25, 26). *S. pneumoniae* is a Gram-positive coccoid bacterium belonging to the *Firmicutes* phylum, while *H. influenzae* and *M. catarrhalis* are Gram-negative bacteria belonging to the class *Gammaproteobacteria* in the *Proteobacteria* phylum.

Streptococcus pneumoniae

S. pneumoniae was first isolated simultaneously in 1880 by the U.S. Army physician George Miller Sternberg and by Louis Pasteur in France (27). Pneumococci are highly virulent, and the leading bacterial cause of death in young children worldwide (28). The clinical manifestations span from sepsis, meningitis and pneumonia to localized upper respiratory infections such as sinusitis and otitis media. *S. pneumoniae* is the leading cause of acute otitis media (25, 29, 30).

Considerable scientific work has been done to investigate the pneumococcal virulence, and the number of potential virulence factors characterized so far have increased remarkably in recent years. The main reason for this interest is the ambition to find new vaccine candidates capable to protect against all pneumococcal serotypes. However, research is also performed in order to clarify pneumococcal pathogenesis, and three important virulence factors, namely the capsule, pneumolysin and autolysin are discussed in more detail below.

Capsule

The polysaccharide capsule is the most important virulence factor in pneumococci. Practically all invasive isolates are encapsulated, although non-encapsulated strains may cause superficial infections such as conjunctivitis (31, 32). There are more than 90 known serotypes whose distribution varies over time and between regions (33). Some serotypes are isolated more often in clinical specimens than others (34). After the introduction of the seven-valent pneumococcal conjugate vaccine, a shift in serotypes causing AOM has been reported (35). In the absence of specific antibodies the capsule is strongly anti-phagocytic by inhibiting complement-

mediated opsonization. The capsule also seems important during colonization and adherence to mucosal cells (36). Children below two years of age have a poor ability to produce antibodies against the polysaccharide capsule (37).

Pneumolysin

Pneumolysin is a crucial virulence factor as shown by the fact that mutated pneumococci devoid of pneumolysin, show markedly reduced virulence in mice compared to their wild-type counterparts (38). It is a toxin that binds to cholesterol in the target cell membrane, where it oligomerizes and forms a pore. In contrast to other cholesterol-depended cytolysins, pneumolysin lacks an N-terminal secretion signal sequence, and therefore depends on bacterial lysis for its release from the cytoplasm (39). However, it has recently been suggested that pneumolysin can be localized and perform its biological activities in the cell wall (40).

At high concentrations pneumolysin is lytic to the human cells (41). At sub-lytic concentrations the toxin exhibits a wide range of effects on the host, some of which are depended on pore formation, whereas others are not (42). Examples include complement activation (43), inhibition of complement-mediated opsonization of the pneumococcus (44), inhibition of ciliary beating by the respiratory epithelium (45), cytokine production via TLR4-stimulation (46), induction of apotosis (47) and activation of intracellular oxygen radical production in neutrophils (48).

Autolysin

Several cell-surface proteins anchor to the pneumococcal cell wall by interactions with phosphoryl choline residues on teichoic and lipoteichoic acids. These choline-binding proteins include the autolysins LytA, LytB and LytC, which are enzymes that decompose the peptidoglycan and are thought to be important during cell wall growth and turnover. LytA is the major pneumococcal autolysin responsible for the spontaneously lysis that occurs when pneumococci reach the stationary phase of growth. The enzyme is an N-acetylmuramoyl-L-alanine amidase which cleaves the bond between N-acetylmuramic acid and L-alanine in the backbone of the cell wall peptidoglycan (49). LytA is synthesized in a low-active E-form which is present only in the cytoplasm (9, 49). How the transmission to the cell wall occurs is still unknown as the enzyme lacks signal sequence (50), but once there, LytA appears to be present solely in an active C-form (51). Why autolysis does not occur until the bacteria reach the stationary phase of growth is not known either (52).

The virulence of autolysin has been shown in mice challenged with mutant pneumococci devoid of autolysin (53). A crucial role for autolysin in the pathogenesis of pneumococcal meningitis, pneumonia and sepsis has also been shown in animal models (54, 55). However, there is also work done that fails to demonstrate any virulence in mice due to autolysin (56).

The exact role of autolysin in pathogenesis is not clear. The benefits from undergoing self lysis may not seem obvious for the individual bacterium. It has been proposed that lysis is important in transfer of genetic material from competent bacteria to noncompetent cells (57). Another theory proposes that autolysin increases virulence by the release of pneumolysin and inflammatogenic cell wall degradation products such as peptidoglycan and teichoic acids (33). Others question the role of autolysin in the release of pneumolysin (42), since autolysin-mutants still release pneumolysin (58), and pneumolysin may be released before generalized cell lysis occur (59). However, recent results from our group indicate that autolysis is, indeed, important for the biological action of pneumolysin (48).

Haemophilus influenzae

Haemophilus influenzae, belonging to the *Pasteurellaceae* family in the *Proteobacteria* phylum, is a Gram-negative, non-motile and rod-shaped bacterium. It was the first free-living organism whose whole genome was sequenced (60). There are two subpopulations of *H. influenzae*, namely encapsulated strains (serotypes a-f), and strains without capsule (non-typeable). Encapsulated *H. influenzae* type b is highly virulent, causing severe meningitis, sepsis and epiglottitis in children. Since the introduction of the conjugated *H. influenzae* type B polysaccharide vaccine, these infections are now practically eliminated in vaccinated populations (61). Non-encapsulated, non-typeable *H. influenzae* causes infections both in the upper respiratory tract, as well as in the lower respiratory tract, for example exacerbations in patients with chronic pulmonary obstructive disease. *H. influenzae* causes 17-52% of AOM cases (30), and the proportion might have further increased coincident with the introduction of the pneumococcal conjugate vaccine to infants (62). Even before the *H. influenzae* type b vaccine was introduced, most otitis media strains were non-encapsulated (30).

The virulence factors of non-encapsuled *H. influenzae* have not been studied as extensively as those of *S. pneumoniae*. Being a Gram-negative bacterium *H. influenzae* possesses LOS in the outer membrane, which is composed of short oligosaccharide chains and lacks O antigen (16). The chemical composition of the *H. influenzae* LOS shows high intra- and inter-strain variability, and the structure seems to shift constantly (13). Host-derived sialylation of LOS has in a chinchilla model been associated with AOM development, and may be important in biofilm formation (15, 63). It has been observed that bacteria can switch between a phosphorylcholine positive and a phosphorylcholine negative phenotype by phase variation (14). The presence of phosphorylcholine residues in the LOS promoted delayed clearance of bacteria from the lungs of mice (16). Expression of phosphorylcholine was also associated with development of otitis media in chinchillas (64).

A highly conserved *H. influenzae* surface lipoprotein, protein D, has been found to impair ciliary function in a nasopharyngeal tissue model (65). *H. influenzae* devoid

of protein D had reduced virulence in an otitis media model in rats (66), and antibodies against protein D are protective in animal models (67). In fact, a novel vaccine with polysaccharides from eleven pneumococcal serotypes conjugated to protein D seems to be protective not only for pneumococcal AOM, but also for AOM caused by *H. influenzae* (68). Non-typeable *H. influenzae* has been shown to survive inside macrophage-like cells in human adenoid tissue (69), and adherence and internalization into monocytic cells may be mediated by protein D (70).

Moraxella catarrhalis

M. catarrhalis is a Gram-negative diplococcus that has been renamed several times due to advances in taxonomic knowledge. The initial generic name was *Micrococcus*, which was changed to *Neisseria*, and further to *Branhamella*, before the current name *Moraxella* was established in 1984 (71). Although somewhat overlooked, *M. catarrhalis* is now established as a respiratory pathogen, causing excerbations in patients with chronic obstructive pulmonary disease as well as AOM in children.

The isolation rates of *M. catarrhalis* in otitis media middle ear fluids differ widely between different geographic areas. Interestingly, increased isolation rates have been reported in some countries, e.g. in Finland, where the incidence increased from 10% to 23% during 15 years (26). In other countries, the isolation rates are only a few percent (30, 72). After introduction of the seven-valent pneumococcal vaccine, nasopharyngeal colonization with *M. catarrhalis* has increased in children with AOM suggesting that AOM due to *M. catarrhalis* might be more common in the future in vaccine-using areas (73).

M. catarrhalis probably lacks capsule, but has LOS on the surface that stimulates immune responses. There are three subtypes (A, B and C) of *M. catarrhalis* based on differences in LOS structures, serotype A being the most prevalent type (74). Serotype A also seems to be more common in children than in adults (75).

Since *M. catarrhalis* is an exclusively human pathogen, there are no good animal models for studying the contribution of different virulence factors to pathogenesis. DNA typing methods have revealed two subspecies of *M. catarrhalis*, one of which is more virulent and termed seroresistant (76). In contrast to the serosensitive subpopulation, these isolates usually show resistance to complement-mediated lysis by human serum and adherence to epithelial cells (77). A surface protein, UspA2, has been found to interfere with the complement system (78, 79).

Defences against pathogens

There are ten times more bacteria than eukaryotic cells residing in the human body. These bacteria are restricted to well-defined compartments, such as the skin, the upper airways, the oral cavity and further down in the gastrointestinal canal. Mucosal-resident bacteria give rise to a constant low-grade inflammation in the mucosa, but they are not eliminated by the immune system. Thus, strains can remain as colonizers for a period, whereafter they are replaced by other bacterial strains. Even potentially dangerous bacteria can colonize the mucosa without causing any harm, and their presence usually passess rather unnoticed by the host. However, if bacteria enter normally sterile sites, such as the middle ear cavity, a strong inflammatory response is evoked, the purpose of which is to eliminate the invading microorganism. AOM is caused by bacteria that ascend from nasopharynx via the auditory tube, also called the Eustachian tube, to the normally sterile middle ear cavity (Figure 2).

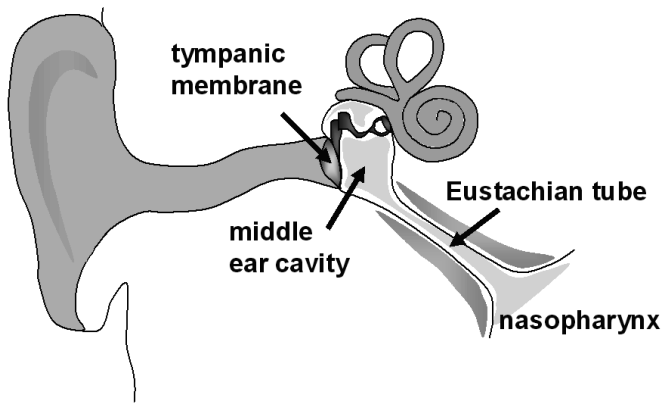

Figure 2: A schematic figure of the normal human ear. The sterile middle ear cavity is connected to the nasopharynx via the Eustachian tube.

Nasopharynx

The nasopharynx extends posterior to the nasal cavity, and superior to the soft palate. It is an airfilled cavity that connects the nose with the pharynx. On each lateral side is the opening of the Eustachian tubes that connect nasopharynx with the cavities of the left and right middle ear, respectively. The nasopharyngeal mucosa contains numerous folds and crypts. The epithelium covering the nasopharyngeal mucosa is of different types and its distribution varies with age. Whereas newborn infants mainly have a pseudo-stratified columnar epithelium,

stratified squamous epithelium dominates in adults (80). There are also considerable amounts of ciliated epithelium and numerous goblet cells between the ciliated cells (80).

Mucosa-associated lymphatic tissue (MALT) is distributed in various regions of the naso-oro-pharyngeal tract and also further down in the gastro-intestinal tract. The nasopharyngeal tonsils or the adenoids are situated in the roof and posterior wall of the nasopharynx, and the tubal tonsils (also known as the *tonsilla tubaria*, Eustachian tonsils, or Gerlach tonsils) are located near the orifice of the Eustachian tube (81).

Bacteria in nasopharynx

The surface of the nasopharyngeal mucosa and the adenoid are colonized by numerous aerobic and anaerobic bacterial species (82, 83). Most bacteria are commensals such as viridans streptococci, *Corynebacterium* spp. and *Neisseria* spp., but there are also potential AOM pathogens including *S. pneumoniae*, non-typeable *H. influenzae* and *M. catarrhalis*. The bacterial community is highly dynamic and a constant turnover of strains occurs (84, 85). In small children the colonization rate of potential bacterial pathogens in nasopharynx is high, whereas the rate decreases by age to a rather low level in adults.

The bacteria have many strategies to remain as colonizers in the nasopharyngeal flora for some time. Colonization by *S. pneumoniae* is facilitated by the pneumococcal capsule, which reduces entrapment in the mucus (36). Whereas the capsule seems advantageous during the first steps of colonization, adherence to and invasion of epithelial cells are associated with loss of capsular material (86). The pneumococci can undergo spontaneous phase variation between an opaque and a transparent colony form (87). The transparent variant is associated with less capsular material and more teichoic acid than the opaque variant (88), and is linked with enhanced ability to colonize nasopharynx (87).

Bacteria adhere to non-ciliated epithelium in the nasopharynx (89), but bacteria have also been found residing intracellularly in the adenoid tissue (69, 90). Bacterial adherence is mediated through interaction of bacterial adhesins to specific host structures in the nasopharynx. Phosphorylcholine is present in the cell wall of several bacteria residing in the upper respiratory tract, including pneumococci and *H. influenzae*, and binds to the receptor for platelet-activating factor (rPAF) on the epithelial surface (91, 92). Attachment of pneumococci to the PAF receptor may result in invasion of epithelial and endothelial cells (93). Several other adhesins and enzymes are thought to increase pneumococcal adhesion and persistence in nasopharynx, including CbpA (also called PspC or SpsA), neuroaminidases and IgA1 protease (33, 94). Non-typeable *H. influenzae* and *M. catarrhalis* also possess a number of proteins that has been suggested to facilitate adhesion to the epithelium

(95-97), and both species have capacities to invade respiratory epithelial cells (98, 99).

Bacteria compete with each other for nutrients and space in the crowded microenviroment of the nasopharynx. For this purpose, bacteria may produce inhibitory substances such as bacteriocins that suppress growth of other species (100). Commensals including viridans streptococci and *Prevotell*a spp. can inhibit *in vitro* growth of respiratory tract pathogens (82). Interestingly, viridans streptococci collected from the tubal orifice seem to be better inhibitors than strains isolated from the adenoids (101).

The Eustachian tube

The auditory tube, also called the Eustachian tube, connects the nasopharynx with the middle ear cavity. It is covered by ciliated epithelium, which prevents pathogens from nasopharynx entering the middle ear. Bacteria are trapped in the mucous layer and transported away by the movements of the cilia. The mucus layer is formed by glycoproteins called mucins, which are produced by epithelial goblet cells and sub-epithelial glands in the tubal mucosa (102). In animal AOM models, the density of the goblet cells, as well as the volume of the glands, increases during infection. This increased secretory capacity of the Eustachian tube remains for months (103).

Children have a shorter and more horizontal Eustachian tube than adults, thus rendering them more vulnerable to invading microbes through the tube (104). By the action of the *veli palatini* muscles, the tube opens during swallowing, thus equilibrating the pressure in the middle ear and providing air to enter the middle ear. The function of the muscle opening is poorer in children than in adults, but improves gradually by increasing age (105, 106). Inadequate ventilation leads to negative pressure in the middle ear, and decreased drainage into the nasopharynx of fluid produced in the middle ear (104) (107). Under normal conditions, sterility of the middle ear cavity is thought to be maintained in part by anti-microbial proteins and peptides, such as lysozyme, lactoferrin and defensins, which protect the auditory tube and the middle ear from invading microbes (108).

The middle ear

The middle ear cavity is located between the tympanic membrane and the Eustachian tube. The mucosal surface is lined by simple epithelium resting on a thin lamina propria, which is adherent to the periostium of the temporal bone. The tympanic membrane consists of strong connective tissue composed of collagen, elastic fibres and fibroblasts, lined by cuboidal epithelial cells on the inside, and a thin layer of epidermis on the outside. There are few goblet cells and no glands in the normal middle ear mucosa.

Innate immunity

Most bacteria are inhibited from entering the middle ear cavity by chemical and mechanical barriers in the nasopharynx and Eustachian tube. However, when this first line of defence is insufficient, the second line of defence, the innate immune system, takes charge. The mammalian innate immune system is an evolutionarily conserved system that reacts rapidly against invading microbes. Activation leads to inflammation, a coordinated defence reaction by which the microorganisms are usually eliminated. Tissue macrophages are key cells that orchestrate the innate immune response to invading microbes in the tissue.

Phagocytosis

Contact with foreign bodies such as bacteria leads to engulfment of the particle, a process known as phagocytosis. Particles are internalized into intracellular vacuoles called phagosomes, which mature into phagolysosomes with killing capacities. The membrane of the initial phagosome resembles the plasma membrane and its interior resembles the extracellular milieu (109). Recent studies have shown that some phagosomal membranes are derived from the endoplasmatic reticulum (ER) and that parts of the ER fuse with the phagosome during or soon after engulfment (110). The phagosome is fused with sorting endosomes (111). In the sorting endosomes, particles intended for recycling are targeted back to the plasma membrane, whereas particles designed for degradations progress to late endosomes, which eventually fuses with lysosomes forming phagolysosomes (112). The phagolysosome is highly acidic, and contains a variety of compounds for killing of microbes, including hydrolytic enzymes, bactericidal peptides such as defensins, and enzymes for generating cytotoxic mediators (109).

Phagocytosis can be initiated by binding of the particle to receptors on the phagocytic surface, but macrophages can also ingest inert particles such as plastic, sand and coal. There are a broad spectrum of receptors participating in particle recognition and engulfment, for example the Mannose receptor that recognizes mannose or fucose residues on diverse microbes (113) and scavenger receptors such as MARCO, which interacts with Gram-positive and Gram-negative bacteria (114).

Phagocytosis is greatly enhanced if the particles are coated with immunoglobulins (Ig) or complement, a phenomenon called opsonization. C3bi is a breakdown product of C3b, which is recognized by the CR3 receptor, while IgG is recognized by the Fcγ-receptors on phagocytes, FcγI, II and III (115). Receptor activation generally results in a large number of events including rearrangement of the actin cytoskeleton and maturation of the phagosome (109).

Inflammatory mediators in the acute inflammatory response

Recognition of microbial structures by specific receptors leads to production of a wide range of inflammatory mediators, such as cytokines and prostaglandins. In fact, the macrophage is the main producer of inflammatory mediators, which are important messenger molecules in the inflammatory reaction. The inflammatory mediators act either locally och systemically in order to produce an optimal response against invading pathogens.

IL-1β and TNF

The pro-inflammatory cytokines IL-1β and TNF are mainly produced by monocytes/macrophages in response to danger signals such as invading bacteria. Production of active IL-1β requires two steps: production of pro-IL-1β, and processing of pro-IL-1β resulting in secretion of active IL-1β. Production of pro-IL-1β occurs in free ribosomes in the cytosol after activation of pathogen recognition receptors. Pro-IL-1β is cleaved by caspase-1 that is activated by inflammasome formation after stimulation by metabolic stress or bacterial components (116). In contrast, pro-TNF is produced by ribosomes bound to the endoplasmatic reticulum and subsequently cleaved on the surface of the cell by a membrane-bound proteolytic enzyme (117).

Quite remarkably, IL-1β and TNF have similar locally and systemically pro-inflammatory effects in the host, though the production and target receptors differ considerably. Both cytokines up-regulate E-selectin and ICAM-1 on endothelial cells which allows neutrophils to slow down and penetrate through the vessel wall to the site of infection (118, 119). They also stimulate production of prostaglandins and nitric oxid (NO) in endothelial cells, which promote dilatation of the blood vessels. Activation of endothelial cells also leads to production of IL-6 and IL-8, as well as initiation of coagulation (120). In high doses, IL-1β and TNF cause local necrosis in the tissue (121). Systemic reactions include induction of fever, production of some, but not all acute phase proteins, cortisol production, altered metabolism and augmentation of neutrophil production in the bone marrow. A few important differences exist between the effects of IL-1β and TNF on the host: TNF, but not IL-1β can activate bactericidal functions in macrophages, whereas IL-1β, but not TNF can activate naïve T cells.

IL-8

IL-8 is a chemokine that attracts and activates neutrophils (122, 123), but has no other known effects. It is mainly produced by monocytes/macrophages in response to bacteria or bacterial products, but can also be synthesized by endothelial and epithelial cells, in particular after IL-1β and TNF stimulation (124). IL-8 binds to the glycocalyx, the carbohydrate-enriched layer of the endothelial cells, where it interacts with neutrophils that have been slowed down due to vasodilatation. Activated neutrophils migrate into the tissue by an IL-8 gradient, leading to accumulation of neutrophils in the infected tissue.

PGE_2

Prostaglandins, leukotrienes and thromboxanes are synthesized from arachidonic acids, which are spliced from the cell membrane by the action of phospholipases. Arachidonic acid is further metabolised by the enzyme cyclooxygenase to generate prostaglandins, such as PGE_2. Production occurs in cells like macrophages or endothelial cells after inflammatory activation. PGE_2 acts on different receptors distributed in different tissues and produce classical inflammatory symptoms like oedema, redness, pain and fever. It relaxes smooth muscle cells resulting in vasodilatation. PGE_2 also cause pain by sensitization of pain receptors to other mediators, such as histamine and substance P (125). In addition to its pro-inflammatory role in acute inflammation, PGE_2 also have anti-inflammatory properties. It decreases activation of neutrophils, and inhibits production of IL-1β, TNF and IL-12 in monocytes/macrophages, while IL-10 production is stimulated (126-128).

IL-6

IL-6 is produced foremost by monocytes/macrophages, but also by other cells like lymphocytes, endothelial and epithelial cells. It is the cytokine that most efficiently induces the acute phase reaction in the liver (129). During the acute phase response, the production of certain plasma-proteins such as C-reactive protein (CRP), LPS-binding protein, complement factors and fibrinogen are enhanced, while the production of other proteins such as albumin are decreased. The acute phase proteins provide help in fighting the invading microorganisms and protect the tissue from injury. Besides IL-1β and TNF, IL-6 also induces fever (129). However, IL-6 also has anti-inflammatory qualities, and inhibits production of IL-1β and TNF directly as well as indirectly through induction of cortisol production (130). This seems to be one of the steps in which IL-6 directs the transition from an innate to an acquired immune response. This involves resolution of the neutrophil infiltrate, replacement of neutrophils by mononuclear cells, activation of T cell functions and promotion of immunoglobulin secretion by B cells (130, 131).

Local effects of inflammatory mediators

By the action of diverse inflammatory mediators, mainly produced by macrophages after recognition of microbial structures, considerable physiologic changes in the blood vessels occur. During the early stages of inflammation the permeability of the local blood vessels increases. This enables plasma proteins to leak out of the vessel and cause oedema of the infected tissue. Anti-microbial proteins, antibodies and complement get access to the infectious area, where they participate direct or indirectly in the elimination of microbes. Antibodies and complement promote phagocytosis of encapsuled bacteria by opsonization of microbes. Activation of the complement cascade leads to formation of a membrane attack complex, which causes lysis and death of Gram-negative bacteria.

Recruitment of neutrophils

A critical event in the defence against pathogens is recruitment of neutrophils to the site of infection (Figure 3). Neutrophils normally constitute 50-75% of the leukocytes in the blood. In contrast to macrophages, the lifetime of neutrophils is short and they only live for a few days in the blood. Upon infection, the production of neutrophils increases in the bone marrow, and large numbers of pre-formed cells are rapidly mobilized into the blood.

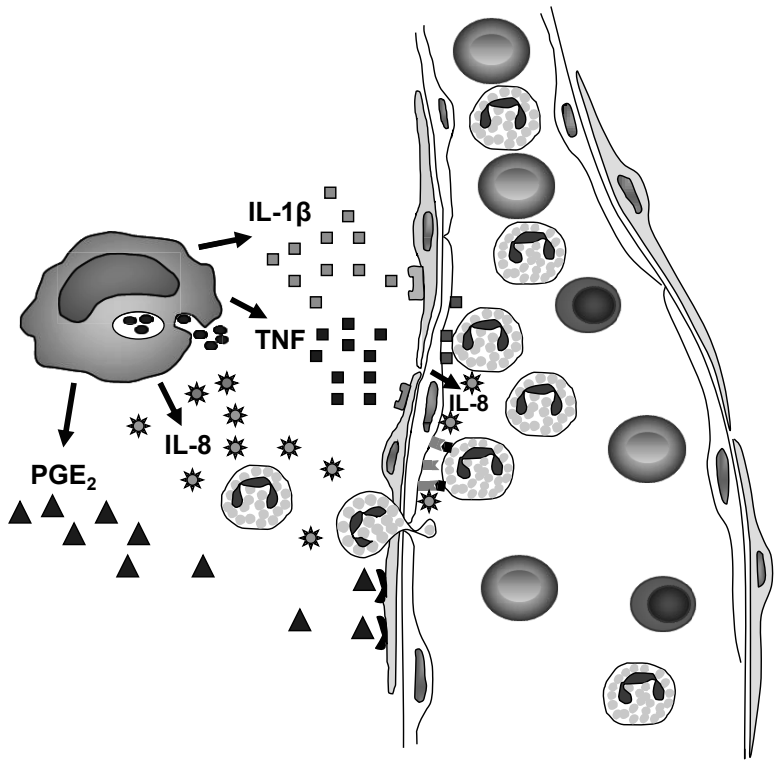

Figure 3: Recruitment of neutrophils to the site of infection by the action of inflammatory mediators mainly produced by tissue macrophages. Due to vasodilatation neutrophils are slowed down and allowed to interact with the endothelium. Following activation the neutrophils are attracted to the infected tissue by IL-8.

Under normal conditions, blood cells travel fast in the middle of the vessel without contact to the endothelium, while the blood plasma flows in the periphery. By opening of precapillar sphincters and relaxation of smooth muscle cells the blood flow in the infected tissue increases. Vessel dilatation due to PGE_2 and NO leads to turbulent blood flow, and the blood cells are slowed down. This allows neutrophils

to interact with endothelial cells which are activated by inflammatory mediators, including IL-1β and TNF (118, 132). Activated endothelial cells express selectins that mediate weak bindings to the neutrophils, resulting in a characteristic rolling and bouncing of the neutrophils upon the endothelial surface. This interaction is followed by a stronger adherence to the endothelial cells via binding to ICAM-1. IL-8, secreted by macrophages and activated endothelium is a strong activator of neutrophils (123). By following a chemotactic gradient of IL-8 into the tissue, the neutrophils are attracted and guided to the infected tissue.

Neutrophils are equipped with numerous granules of four different types: the specific granule, azurophilic granule, gelatinase granule and secretory vesicles. The contents differ between the granules, and there are also differences in the degree of stimuli that is required for secretion of the contents. The secretory granules contain integrins and complement receptors that are mobilized to the plasma membrane when neutrophils encounter endothelial cells. Integrins mediate adhesion to ICAM-1 on endothelial cells, while complement receptors is involved in phagocytosis by recognizing opsonized microbes.

Neutrophils are very efficient in phagocytosis and intracellular killing of bacteria. Granules containing antimicrobial substances and enzymes are recruited to the phagosome. The most abundant granules in the neutrophils are the specific granules which contain the iron-binding protein lactoferrin and lysozym that degrades peptidoglycan. The specific granules also contain membrane-bound NADPH-oxidase, which generates reactive oxygen species, central in microbial killing of neutrophils (133). If the elimination of bacteria has been successful, resolution of the neutrophil infiltrate occurs. Programmed cell death, apoptosis, is induced in the remaining neutrophils, wherafter they are cleared away by tissue macrophages.

Tissue damaging mechanisms

Macrophages and neutrophils possess microbicidal agents that are potential dangers to the host. These agents include free radicals, such as O_2^- and HOCl, which may be released by the neutrophils during killing of extracellular bacteria. Tissue damaging agents may also be released by necrosis of phagocytoc cells during infection. Other potential threats to the host include different proteolytic enzymes. Neutrophils possess serine proteases that mediate bacterial killing, but are also involved in extracellular matrix degradation during infection (134). Matrix metalloproteinases are enzymes produced by neutrophils and macrophages that participate in breakdown of the extracellular matrix (135). Synthesis of metalloproteinases is induced by IL-1β and TNF (135).

Pathogen recognition receptors

Central for innate immune activation during infection is recognition of microbial structures by different receptors on phagocytes. Conserved structures of the microbes, called pathogen-associated molecular patterns (PAMPs), are recognized by innate immune cells that possess different pattern recognition receptors (PRRs). Not only microbe-derived but also endogenous danger signals that are exposed during stress or necrosis of host cells are recognized by PRRs. Macrophages posses a wide range of PRRs. In recent years, extensive work has been performed to elucidate the roles of Toll-like receptors (TLRs) and nucleotide oligomerization domain-like receptors (NLRs) in inflammation.

Toll-like receptors

The Toll-like receptors (TLRs) are a group of mammalian proteins that are homologous to the insect Toll proteins. Each TLR member senses a unique or a limited set of microbial motifs, which usually are essential for the survival of the microorganism, thus preventing the microbe to escape detection by the immune system. There are 10 TLRs described in humans so far (136). The TLRs are membrane-spanning proteins located on the surface of the cell or in the lumen of intracellular vesicles such as endosomes. TLR2 is expressed on the surface of phagocytic cells, but may also be recruited into phagosomes (137). In contrast, TLR9, which recognizs unmethylated bacterial DNA, is solely expressed in intracellular compartments (138). The TLRs are composed of three domains, an outer Leucin-rich repeat domain (LRR) domain, which senses the specific PAMP, a transmembrane domain, and an intracellular effector domain (136). Sensing of the PAMPs is believed to be direct, or more likely, indirect via certain adaptor proteins. The TLR ligands induce dimerizations of the TLRs, forming homodimers (such as TLR4-TLR4) or heterodimers (such as TLR2-TLR1 or TLR2-TLR6) (139). Signalling through TLRs activates downstream events resulting in production of inflammatory mediators.

TLR2

TLR2 has been thought to recognize a wide range of PAMPs from various pathogens (140). However, this highly diversity of agonists have been questioned. It is generally accepted that lipoproteins/lipopeptides are sensed by TLR2, and perhaps also peptidoglycan (141, 142), whereas other described agonists may be falsely identified due to lipoprotein contamination (143, 144). Ligand recognition leads to activation of the MyD88-dependent pathway. A series of down-stream events results in activation of mitogen-activated protein kinases (MAPKs) and nuclear factor-κB (NF-κB). Activated MAPKs and NF-κB promote transcription of numerous genes involved in the immune response such as pro-inflammatory cytokines, anti-microbial peptides, adhesion molecules and MHCs (145). TLR2 is thought to be particularly important in the defence against Gram-positive bacteria (146-148). TLR2 deficient mice show higher bacterial loads in the brain and

increased disease severity compared to wild-type mice in a pneumococcal meningitis model (149, 150). Moreover, clearance of colonization by *S. pneumoniae* in nasopharynx was shown to depend on TLR2 (151). However, bacterial clearance was unaltered in TLR2 deficient mice compared to wild-type in experimental pneumococcal pneumonia (152).

TLR4

TLR4 is activated by LPS, a major constituent of the outer membrane of Gram-negative bacteria. LPS binds to LPS-binding protein (153), and the complex is recognized by CD14 on macrophages (154). TLR4 associates with the LPS-LPS-binding protein complex via the co-receptor MD-2 (155). Stimulation of TLR4 leads to activation of two different pathways: the MyD88-dependent pathway, and the TRIF-depended pathway. Both pathways lead to activation of NF-κB and MAPK, and subsequent transcription of inflammatory cytokines. The TRIF-depended pathway also leads to activation of the transcriptor factor IRF3, which induces production of type I interferons (136). The massive immune response to LPS may be a consequence of the ability of LPS to activate both pathways. Other structures than LPS have also been reported to activate TLR4, such as the *S. pneumoniae* toxin pneumolysin (46). TLR4 is localized on the surface of phagocytes, but has also been found intracellular in dendritic cells (156).

The NLR family

Presently, there are more than 20 described members in the human NOD-like receptor (NLR) family (157). Common to all NLRs, there is a C-terminal ligand-sensing LRR domain, a central nucleotide-binding NACHT domain that mediates oligomerization of the NLRs upon activation, and a variable N-terminal effector domain, which can be a pyrin domain (PYD), a CARD or a BIR domain. Most NLRs also contain a NACHT-associated domain (NAD) (116). Evidence of direct binding of the ligand to the LRR is still lacking. Based on the class of the N-terminal domain, the NLRs are further divided into subfamilies, of which the PYD-containing NALPs and the CARD-containing NODs are the largest subfamilies (Figure 4). Upon activation of NLRs, a conformation change occurs that enables the NACHT regions to oligomerize with corresponding NACHT regions on similar receptors, forming an active multimolecular complex. NLRs are found in immune cells including phagocytes, but also in e.g. epithelial cells (116). Characteristically, the receptors are located intracellular in the cytosol. Of particular interest are the NOD1 and NOD2 receptors, which sense peptidoglycan motifs, and NALP3, which promote inflammasome formation and IL-1β secretion.

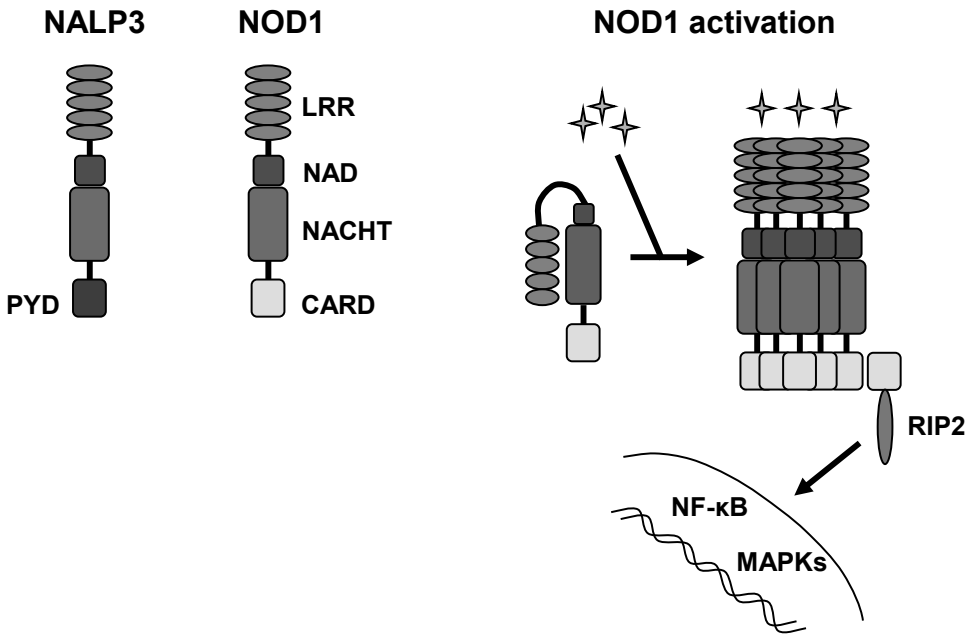

Figure 4: Schematic figure of two NOD-like receptors (NLRs), NALP3 and NOD1. Upon activation of NOD1 or NOD2, a conformation change occurs, which enables the protein to oligomerize. Recruitment of RIP2 results in activation of NF-κB and MAPKs and subsequent transcription of genes encoding inflammatory molecules including cytokines.

NOD

NOD1 and NOD2 sense distinct structures from bacterial peptidoglycan present in the cytosol. Upon ligand recognition, a conformation change of the receptor occur which enables several receptors to oligomerize. The N-terminal CARD domains recruits the kinase RIP2 through CARD-CARD interactions (158-160). Oligomerization of RIP2 results in a number of down-stream events, which results in activation of NF-κB and MAPKs and subsequent transcription of pro-inflammatory cytokines (158). While NOD1 is expressed in numerous kinds of cells, NOD2 is thought to be restricted to fewer cell types (157). However, NOD2 has been found both in immune cells such as macrophages (161), as well as in other cells like the oral cavity epithelial cells (162).

NOD1

NOD1 recognizes peptidoglycan that contains *meso*-DAP, a compound in the stem peptide of Gram-negative bacteria (Figure 1) (163, 164). Indeed, NOD1 detects a *meso*-DAP containing muramyl tripeptide, which is a peptidoglycan degradation

product released during the metabolism of Gram-negatives (6). Interestingly, some Gram-positive bacteria, including *Listeria monocytogenes* and *Bacillus cereus* contain *meso*-DAP in their peptidoglycan as well (6).

NOD2

NOD2 detects muramyl dipeptide (MDP), a molecular motif present in both Gram-positive and Gram-negative bacteria (Figure 1) (165, 166). NOD2 can also detect a L-Lysin containing muramyl tripeptide, which is present only in Gram-positives (167). Mutations of NOD2 have been associated with Crohns disease, which is characterized by chronic inflammation in the intestine (157).

The NALP3 inflammasome

NALP3 (also known as NLRP3) is expressed in various cells such as phagocytes and oral epithelial cells (168). Activation of NALP3 leads to formation of a multiprotein complex, termed inflammasome, which is necessary for activation of caspase-1 and subsequent IL-1β secretion (Figure 5) (169). NALP3 is present in the cytosol in an auto-inhibited state. Upon activation, oligomerization of NALP3s occurs by NACHT-NACHT interactions in an ATP-depended way (170). The adaptor protein ASC is then recruited to the complex. ASC contains a PYD that binds to the corresponding PYD of NALP3. ASC also contains a CARD domain which recruits caspase-1 to the complex by CARD-CARD interactions (116). Activated caspase-1 cleaves pro-IL-1β into active IL-1β which is then secreted out of the cell (171, 172).

The exact mechanism of NALP3 activation is not known, but a large number of compounds have been shown to induce inflammasome formation *in vitro* (116). These include microbial structures such as the peptidoglycan degradation product MDP, bacterial toxins and viral DNA. Endogenous signals, such as ATP and uric acid released by injured or stressed cells, also activate caspase-1 leading to IL-1β secretion. Uric acid is secreted from necrotic cells and forms urate crystals in the extracellular milieu, which is the compound responsible for NALP3 activation. In addition, asbestos particles have been shown to stimulate inflammasome formation. Taken together, it is believed that the main function of the NALP3 inflammasome is to react on various danger signals.

The diversity of inflammasome activators has raised the question of a common downstream signal. Spontaneous formation of the inflammasome occurs when intracellular potassium is lowered (173). Several (but not all known) activators of NALP3 can trigger K^+ efflux (174). Moreover, most of the bacterial toxins that activate NALP3 are pore-forming toxins, which trigger K^+ exit from the target cell. Another potential common mechanism is the production of reactive oxygen species, which occurs after stimulation by various activators, such as asbestos, urate crystals and ATP (175-177).

Another theory suggests that microbial structures activate NALP3 directly or indirectly in the cytosol. How microbial products translocate from the phagocytic endosomes to the cytoplasm is not clear. It has been proposed that microbial compounds are delivered to the cytosol through the pannexin-1 channel or through bacterial toxin-forming pores (157, 178). Stimulation of NALP3 by ATP is associated with activation of the purinoreceptor P2X7 that interacts with the pannexin-1 channel (178, 179). It has also been suggested that inflammasome formation requires two separate signals, one provided by microbial products, the other by extracellular ATP or pore-forming bacterial toxins (157).

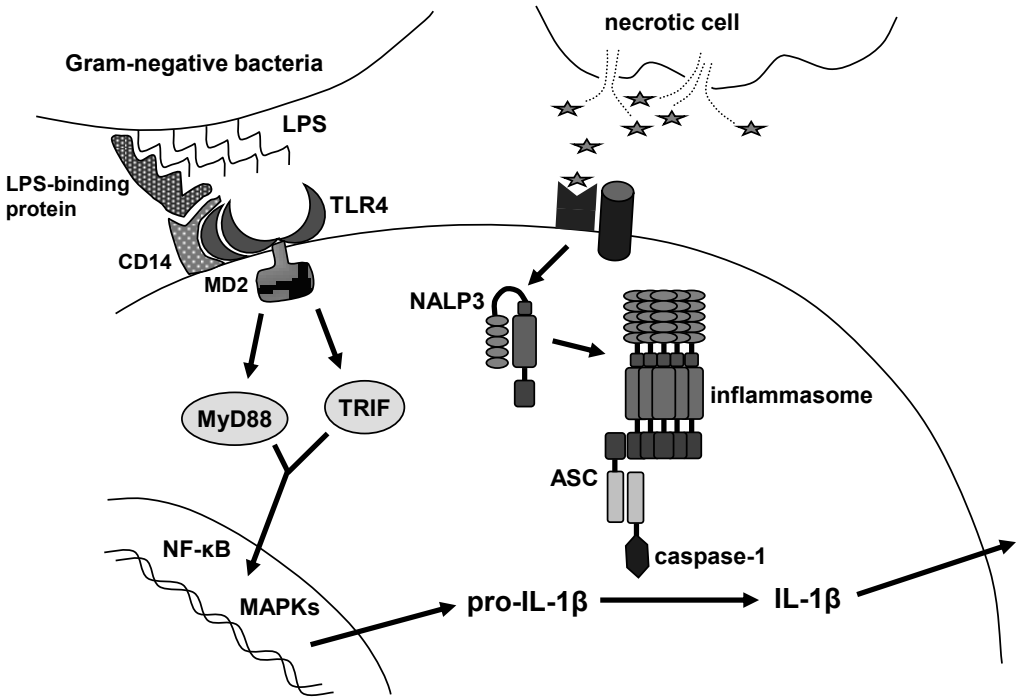

Figure 5: Production of IL-1β requires two signals, one that results in production of pro-IL-1β and another leading to caspase-1 activation and cleavage of pro-IL-1β to active IL-1β. In this example the production of IL-1β is initiated by indirect recognition of LPS by TLR4, which results in activation of NF-κB and MAPKs. Endogenous danger signals released by necrotic cells are sensed by NALP3 whose activation leads to inflammasome formation and activation of caspase-1.

Table 1**:** Some macrophage receptors and their activators

Receptor family	Receptor	Localisation	Examples of activators	Examples of resulting event
C-type lectin receptors	Mannose	Cell surface	Mannose, fucose	Phagocytosis
Scavenger receptors	MARCO	Cell surface	Large negatively charged molecules	Phagocytosis
TLR	TLR2	Cell surface, endosome	Lipoproteins	Production of inflammatory mediators
	TLR4	Cell surface, Intracellular compartments	LPS	Production of inflammatory mediators
	TLR9	Intracellular compartments	Unmethylated bacterial DNA	Production of inflammatory mediators
NLR	NOD1	Cytosol	*Meso*-DAP in Gram-negative peptidoglycan	Production of inflammatory mediators
	NOD2	Cytosol	MDP in peptidoglycan	Production of inflammatory mediators
	NALP3	Cytosol	MDP, Urate crystals, ATP	IL-1β, IL-18 secretion

Acquired immunity

When the innate immune system is insufficient for defeating infectious agents, the acquired immune system provides help in the defence. Compared to the innate immune system, activation of the acquired immune system is a slower process, and it may take weeks to fully develop an aquired immune response the first time an antigen is encountered. However, in contrast to the innate response, acquired immunity is equipped with memory, and activation occurs much faster when encountering the same microbe repeatedly. Lymphocytes are organized in secondary lymphoid organs where they are presented for their specific antigens. In the upper respiratory tract, different tonsillar tissues constitute the mucosa-associated lymphatic tissue (MALT).

The tonsils are highly organized structures with different compartments containing dendritic cells, T cells and B cells (180). There a numerous crypts, which increase the contact considerably. Primed B cells are distributed to secretory tissues where they differentiate into plasma cells that produce IgA and IgM (181). There is evidence that B cells are directed specifically from nasopharyngeal MALT to the mucosa of the upper respiratory tract, a process known as homing (182).

T helper cells and the diversity of aquired immune responses

Aquired immune responses start when microbes or other antigens are engulfed and processed by dendritic cells strategically positioned in the skin and mucosa. The antigen-presenting cells migrate to a lymph node, and present their antigen on MHC class II molecules for circulating $CD4^+$ lymphocytes. Activated $CD4^+$ T cells mature into T helper cells or regulatory T cells. Besides antigen recognition $CD4^+$ T cells must receive co-stimulatory signals for activation. The co-stimulatory factors are provided by activated antigen-presenting cells and include accessory surface molecules, as well as T cell activating cytokines such as IL-1β and IL-12. This is a security system to confirm that the T cell is reacting on a relevant antigen which already has activated innate immune cells. Recognition of antigen without co-stimulatory signals results in anergy, a state of immune unresponsiveness. Based on the conditions provided during antigen presentation, the nature of the antigen and the antigen-presenting cell, different reaction patterns of the $CD4^+$ T cells develop. Somewhat simplified, $CD4^+$ T helper cells can be said to promote and control three different events; macrophage activation, antibody production and $CD8^+$ T cell activation.

T helper cells stimulate B cells to mature into antibody secreting plasma cells. The T cell derived cytokines IL-4, IL-6 and IL-10 are important for this B cell maturation. Also cytotoxic $CD8^+$ T cells mature under the influence of cytokines from T helper cells, mainly IL-2 and IFN-γ. Activated $CD8^+$ T cells recognize their

specific antigen on MHC class I molecules, whereby the cell presenting the antigen is killed.

Macrophage activation by T helper cells

Macrophage activation is important to promote killing of ingested bacteria. IFN-γ, produced by activated T helper cells, increases macrophage bactericidal functions by production of free radicals and lysosomal enzymes and also enhances MHC class II expression. T helper cell promotion of the killing capacity of macrophages is called cell mediated immunity.

IL-12 is composed by the subunits p40 and p35 that form the biological active p70 heterodimer (183). Whereas p35 is produced ubiquitously and constitutively at low levels, p40 expression is restricted to phagocytes and antigen-presenting cells. Hence, the IL-12 heterodimer is mainly produced by monocytes, macrophages and dendritic cells in response to bacteria (184). IL-12 induces proliferation, IFN-γ production and increased cytotoxic activity of T cells and NK cells (185). IFN-γ, in turn, promotes further production of IL-12 from monocytes and activates bactericidal functions of macrophages in synergy with TNF (Figure 6) (186, 187). IL-12, IFN-γ and TNF are therefore key cytokines in cell-mediated immune reactions, and IL-12 also promotes T cell differentiation into T helper 1 cells (185).

IL-10, produced by monocytes/macrophages and T cells down-regulates macrophage activation and antigen presentation, and inhibits production of IL-12 and IFN-γ. Instead, IL-10 stimulates B cell proliferation and immunoglobulin secretion, and enhances cytotoxic T cell development (188, 189). IL-12 and IFN-γ production is also potently inhibited by IL-10 and PGE_2 (190, 191).

Since long, the phagocyte-activating cytokines IL-12, IFN-γ and TNF have been known to protect against intracellular bacteria, such as *Listeria monocytogenes* (192). Individuals with deficiencies in the IL-12/IFN-γ loop are especially susceptible to infections with intracellular bacteria including non-tuberculous *Mycobacteria* and *Salmonella* spp. (193). However, in recent years it has become evident that these cytokines are important for the defence against extracellular bacteria, including *Staphylococcus aureus* (194), group B streptococci (195) and *S. pneumoniae* as well (196, 197). A potential role for IL-12/ IFN-γ in probiotic treatment has also been suggested, since *Bifidobacteria* and *Lactobacillus* spp., commonly used as probiotic strains, are strong inducers of IL-12 and IFN-γ (198, 199).

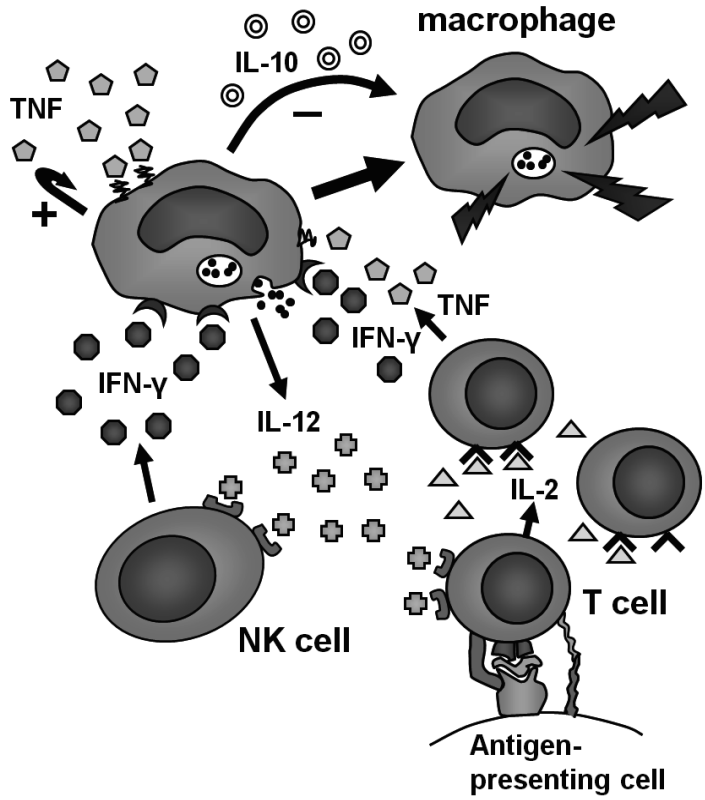

Figure 6: Macrophage activation by T helper cells. IL-12 stimulates T helper cells and NK cells to produce IFN-γ, which enhance bactericidal functions in macrophages in synergy with TNF. Macrophage activation is potently inhibited by IL-10.

Induction of different cytokines by bacteria *in vitro*

Analyses of inflammatory mediators produced by human mononuclear cells in response to different bacteria have revealed an interesting pattern: Intact Gram-positive bacteria induce much more IL-12, IFN-γ and TNF than do intact Gram-negative bacteria, which instead induce more IL-6, IL-8, IL-10 and PGE_2 than do Gram-positives (Figure 7) (200-202). Furthermore, the same type of response as that to intact Gram-negative bacteria could be induced using pure LPS, whereas no soluble components of the Gram-positive cell wall induces IL-12 or comparable

levels of TNF or IL-1β as do intact bacteria (Figure 7) (200, 201). The requirements for IL-12 production in response to the Gram-positive bacteria were investigated using *Bifidobacteria adolescentis* as model bacterium. Phagocytosis was obligate to obtain an IL-12 response, since cytochalasin treatment abrogated production of IL-12 but not IL-6 (199). Moreover, inhibition of the NF-κB pathway reduced the production of IL-12 (199). Soluble bacterial fragments, or peptidgoglycan, lipoteichoic acid or muramyl dipeptide (MDP) did not induce any IL-12, as previously shown, and actually inhibited the response to intact bacteria (199).

The above mentioned studies of the cytokine pattern in reponse to different Gram-positive and Gram-negative bacteria included 14 different bacterial species. The seven Gram-positive bacteria belonged to the *Actinobacteria* and the *Firmicutes phyla*, the only ones containing Gram-positive bacteria. Six of the seven Gram-negative species investigated belonged to the *Proteobacteria* phylum, while one, *Veillonella parvula*, belonged to the *Firmicutes* phylum. Interestingly, *V. parvula* actually induced IL-12 in levels almost comparable with those induced by the Gram-positive bacteria, which raised the question whether Gram-negative bacteria, belonging to the "Gram-positive" *Firmicutes* phylum behave like Gram-positive bacteria in means of cytokine induction in general and IL-12 in particular. Also, it remains to be shown that this cytokine pattern induced by different Gram-positive and Gram-negative species is conserved when bacteria from other phyla are investigated.

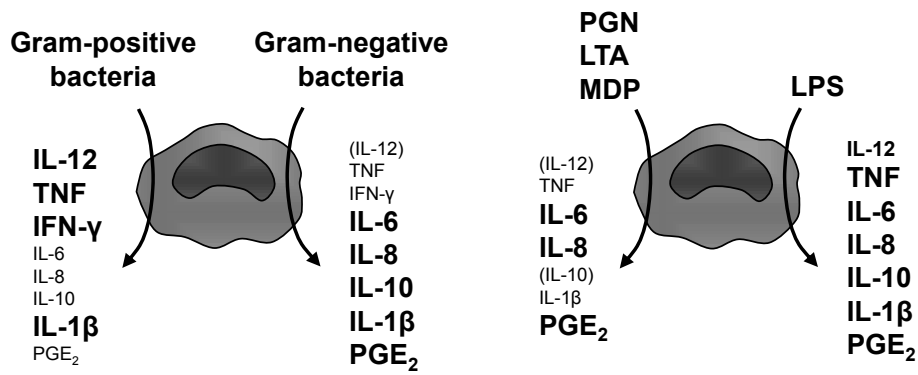

Figure 7: Different patterns of inflammatory mediators induced by Gram-positive and Gram-negative bacteria, respectively, in human monocytes. While the levels of inflammatory mediators induced by Gram-negative bacteria could be mimicked by using pure LPS, soluble fragments from the Gram-positive cell wall were poor inducers of cytokines that were induced in high levels by intact Gram-positive bacteria.

Acute otitis media

Acute otitis media (AOM) is the most common bacterial infection in childhood. Children between 6 and 11 months are at highest risk, and by age three years every second child has experienced one episode of AOM (203). In some children AOM tend to relapse repeatedly. In Sweden, an otitis-prone child is defined as one who has had six or more AOM episodes before the age of two years. In other countries, the definition is more inclusive leading to a higher proportion of otitis-prone children in the population. The symptoms of AOM vary with age. When children between 2 and 24 months were examined, the most common symptoms of AOM were those typical of a viral upper respiratory infection, i.e. runny nose and/or cough (204), which relates to the fact that most cases of AOM develop in conjunction with a viral upper respiratory infection (see below). Other symptoms and signs often present in AOM are ear ache, fever, redness of the tympanic membrane and purulent middle ear fluid (204).

Risk factors

A number of risk factors for AOM have been described, such as young age, day care attendance, siblings, male gender, passive smoking and winter season (107). Most of these conditions predispose children to upper viral respiratory infection, which is considered the most important predictor for AOM in children (107). It has been estimated that AOM occurs in approximately 20% of children with upper respiratory infections (107), usually 3-4 days after the onset of the viral infection (205, 206). Infection with respiratory syncytial virus (RSV) has been associated with the highest risk of developing AOM (207, 208). Other viruses associated with AOM include adenovirus, coronavirus, influenza virus and rhinovirus (209).

Pathogenesis

AOM is caused by bacteria that ascend into the middle ear from nasopharynx via the Eustachian tube. Viral infections promote adherence and colonization of the nasopharynx by pathogenic bacteria (107, 210), perhaps by providing molecules on the epithelial cells which are used as receptors by the bacteria (211). Infection with influenza A also results in Eustachian tube dysfunction with damage and disappearance of ciliated cells, accumulation of secretion in the lumen and negative pressure in the middle ear (212, 213). Moreover, intranasal inoculation with influenza A, but not pneumococci, induces neutrophil dysfunction in chinchillas (212). In a chinchilla model, otitis media developed in 4% of the animals inoculated with influenza virus alone, and in 21% inoculated with *S. pneumoniae* alone, whereas 69% of the animals inoculated with both virus and bacteria developed otitis media (214).

The main bacterial AOM pathogens, *S. pneumoniae*, Non-typeable *H. influenzae* and *M. catarrhalis* are more often isolated from nasopharynx in children prone to

otitis media compared to control children (82, 215, 216). Children with increased risk of AOM development also seem to have an unbalanced commensal nasopharyngeal flora with fewer viridans streptococci and anaerobic bacteria such as *Prevotella* spp. with interfering capacities against bacterial pathogens (82, 217, 218). Interestingly, healthy children have higher levels of pro-inflammatory cytokines in nasopharynx than children with recurrent AOM (219). There are some indications of immune activation in children colonized with *H. influenzae* as they have higher levels of cytokines in nasopharynx compared to non-colonized children (219). Moreover, mouse experiments have shown that colonization with *S. pneumoniae* results in influx of neutrophils into the mucosa (36). However, this inflammatory response is insufficient for bacterial clearance and the bacteria therefore remain in the nasopharynx (36). The high incidence of potential bacterial pathogens in children might explain the observation that healthy children have higher levels of IL-1β, TNF and IL-6 in nasopharyngeal secretions compared to healthy adults (219).

Ascending of microbes into the middle ear cavity is associated with a strong inflammatory response. Activation of the defence systems lead to secretion and accumulation of fluid in the middle ear that contains large amounts of inflammatory mediators and immune cells such as neutrophils (220). In the early phase of infection the fluid in the middle ear is mainly derived from serum, whereas the purulent otitis media exsudate contain locally synthesized components as well (221). During infection the number of goblet cells increases and gland formation occurs (222, 223). Animal experiments show that an enhanced secretory ability of the middle ear mucosa persists six months after a single AOM episode (222).

Pneumococcal AOM is associated with more severe symptoms such as fever and ear ache compared to AOM caused by *H. influenzae* or *M. catarrhalis* (204, 224), whereas *H. influenzae* often cause bacterial conjunctivitis in conjunction with AOM (204). AOM due to *H. influenzae* often affect somewhat older children than otitis media caused by *S. pneumoniae* or *M. catarrhalis* (26, 204), and non-typeable *H. influenzae* is also more commonly isolated from children with recurrent AOM compared to the other middle ear pathogens (26, 225). However, there are considerable overlaps between the clinical presentations of AOM and the bacterial etiology, and the only way to verify a microbial agent in AOM is to perform tympanocentesis.

Sometimes spontaneous perforation of the tympanic membrane occurs. *S. pneumoniae* is more often cultured from spontaneous perforated AOM than the other pathogens (204, 226). *S. pneumoniae* may also cause acute mastoiditis, which is a serious infection in the temporal bone of the skul, and a rare complication in Sweden (227).

Cytokines in middle ear fluids

The presence of cytokines in relation to bacterial pathogens in middle ear fluid has been studied. Middle ear fluid was collected after tympanocentesis, and patients with tympanostomy tubes or spontaneous perforation of the tympanic membrane were excluded. Interestingly, the levels of IL-1β, TNF, and IL-8 were significantly higher in middle ear fluids positive for bacterial pathogens by culture compared to culture-negative samples (228-230). In contrast, no significant differences in IL-6 levels between culture-positive and culture-negative samples could be shown (231). After 3-4 days the levels of IL-1β were significantly decreased irrespective of bacterial findings in the fluid (228).

No previous study has related cytokine levels in middle ear fluids to bacteria detected by PCR. Nor have inflammatory mediators been measured in middle ear fluids from AOM patients with spontaneous perforation of the tympanic membrane.

Treatment

Treatment recommendations against AOM vary worldwide. In Sweden, children under the age of two years are always recommended antibiotic treatment. Since AOM show high rates of spontaneous resolution, expectation can sometimes be sufficient in children above two years. However, antibiotic treatment is indicated when the tympanic membrane has ruptured.

The role of viruses in AOM

There is no question that viral infection paves the way for bacterial otitis media, but whether the presence of viruses in the middle ear fluid plays a role in the pathogenesis is less clear. By using culture and/or antigen detection, virus has been detected in in approximately 20% of middle ear fluids from AOM children (107). With PCR techniques, this rate increases considerably (232). However, the clinical significance of virus detected only by PCR is questioned, as it may be "too sensitive" a technique. For example, the cytokines IL-1β, TNF, IL-6 and IL-8 are all markedly elevated in nasal secretions in children during acute viral respiratory tract infections (233). The cytokine levels correlate with the severity of symptoms (234, 235), and nasopharyngeal IL-1β levels have been reported to be higher in children who developed AOM during upper respiratory tract infections compared to those who did not (236). IL-1β and IL-6 levels were also higher in infectious nasopharyngeal secretions containing virus detected by conventional culture and antigen tests compared to virus detected by PCR technique (236). Similarly, AOM development during viral upper respiratory tract infection correlated better with viral findings using culture than PCR (209).

Viruses are sometimes detected in AOM effusions without evidence of concomitant bacterial presence (237). Whether they can cause AOM by themselves is not

known. An alternative explanation is that the bacteria causing the AOM episode are dead and therefore not detected by culture.

Viruses may prolong the duration of symptoms and prevent resolution of AOM. Associations between treatment failure of bacterial AOM and the presence of virus in middle ear fluid have been reported (238, 239). Penetration of antibiotics into the middle ear cavity in chinchillas was reduced in viral and mixed viral-bacterial infections compared to infections caused by bacteria alone (240), and the same tendency was seen in children with AOM (241). Histamine, IL-8 and leukotriene B4 have all been measured in higher amounts when virus coexists with bacteria in middle ear fluids (242, 243). The presence of virus in the effusion might stimulate local production of inflammatory mediators, which could contribute to the prolonged symptoms and reduced concentrations of antibiotics seen in middle ears infected with both virus and bacteria.

Secretory otitis media

Secretory otitis media (SOM), also called otitis media with effusion, is defined as the presence of fluid in the middle ear without signs or symptoms of an acute infection (244). The inflammatory cells in the middle ear effusion are dominated by macrophages, reflecting the chronic condition of secretory otitis media (104). Persistent middle ear fluid may lead to hearing loss due to decreased mobility of the tympanic membrane. In children this may affect language development and behaviour. SOM may appear spontaneously due to poor Eustachian tube function, or as a sequel to AOM (245). About 90% of children have experienced at least one episode of SOM before school age, most often between ages 6 months and 4 years (245). The condition most often resolves spontaneously, but 5-10% remains one year or longer (245). Children with SOM are generally observed clinically for three months or longer before interventions are considered, usually insertion of tympanostomy tubes during general anaesthesia.

Longstanding SOM impose morphological changes in the middle ear mucosa. Goblet cells proliferate and their secretiory activity is enhanced. Mucous glands may appear (222, 223), and the gland density may be high in long-standing SOM (246).

By using PCR, bacterial DNA from *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* are detected in up to 80% of the SOM cases (247, 248). As the detection rate of bacteria by culture usually is strikingly lower in SOM than in AOM, and as SOM is rather unresponsive to antibitotic treatments, a biofilm hypothesis has been put forward. A biofilm is a highly organized network of aggregated bacteria surrounded by an extracellular matrix that is usually adherent to a surface (249). Biofilms provide resistance to host defences such as opsonization and phagocytosis (250). There is some evidence of biofilm formation

in the middle ear mucosa of children with longstanding SOM as well as in children with recurrent otitis media including cases where culture was negative and PCR positive for a bacterial pathogen (251, 252). *S. pneumoniae*, *H.influenzae* and *M. catarrhalis* all have the capacity to form biofilms *in vitro* as well as *in vivo* (249). Bacteria appear to have certain phenotypic characteristics when living within a biofilm. Sialylation of LOS *H.influenzae* has been found to promote biofilm formation (15). Moreover, the expression of phophorylcholine in LOS might influence the early inflammatory response and biofilm formation upon ear infection with *H. influenzae* (253). Since experimental bioforms a formed rather rapidly after bacterial challenge, biofilms might be present also in acute otitis media (249).

Probiotics in upper respiratory tract infections

Probiotics are defined as "live microorganisms which when administrated in adequate amounts confer a health benefit on the host" (254). Probiotics have been used as dietary supplements for decades, and many human studies have tested the efficacy of probiotics in various conditions, foremost diseases in the gastrointestinal tract (255). However, less is known about the role of probiotics in upper respiratory tract infections. In a recent systematic review regarding the effects of probiotics, it was concluded that orally administered probiotics may reduce the severity of symptoms in respiratory tract infections, but not necessarily reduce the incidence of diseases (256). However, long-term dietary ingestion of probiotics was showed to reduce both the incidence and duration of cold and influenza-like symptoms in children (257).

Different species of the *Lactobacillus* genera are among the most common bacteria used in probiotics. Although the probiotics are administered orally in most studies, there are a few investigations in which probiotic bacteria are applied locally by spray treatment. By this regime, viridans streptococci have been shown to reduce recurrences of pharyngotonsillitis due to group A β-hemolytic streptococci (258, 259). Nasal spray treatment with viridans streptococci also reduced the recurrence rate of AOM in children. Moreover, fewer children in the spray treatment group developed SOM in the aftermath of infection compared to children receiving placebo (260). The study did not investigate whether spray treatment has any effect on the middle ear fluid in established secretory otitis media.

AIMS

The aims of this thesis were:

- to investigate whether the dichotomy in cytokine pattern in response to Gram-positive and Gram-negative bacteria follows or crosses taxonomic borders.
- to study whether autolysis of the Gram-positive bacterium *Streptococcus pneumoniae* influences the cytokine levels induced by intact bacteria.
- to investigate if the cytokine response in middle ear infections differs between infections caused by Gram-positive and Gram-negative bacteria, respectively.
- to study if nasal spray treatment with certain Gram-positive bacteria can induce clinical recovery in patients with secretory otitis media.

MATERIAL AND METHODS

Bacteria (I, II, III)

To study cytokine responses to different Gram-positive and Gram-negative bacteria in relation to taxonomic relatedness, 14 Gram-positive and 23 Gram-negative bacterial species from five phyla of medical interest were used. The strains were derived from different culture collections (ATCC, CCUG and VPI) and are all listed in Table 1, paper I. Seven strains of *L. monocytogenes* of different serovars were also used; four of which were human clinical isolates and three of food related origins. In addition, a *S. mitis* strain (CCUG) was used as a Gram-positive control. The strains were cultured on suitable media under optimal conditions, washed and adjusted to the same concentration according to a protocol used earlier with minor modifications (198). Thereafter, the bacteria were UV-inactivated, which was confirmed by negative viable count. The *S. pneumoniae* (n=3), *H. influenzae* (n=3) and *M. catarrhalis* (n=1) strains used for stimulation of human monocytes *in vitro* (paper III) were all isolated from AOM patients in the same study. These clinical strains were prepared similarily as the bacteria described above.

In paper II, the cytokine production was studied in response to intact pneumococci and pneumococci allowed to autolyse. For this purpose the virulent strain *S. pneumoniae* D39 (D39 WT), its pneumolysin-deficient mutant (D39 PLY-) and autolysin-deficient mutant (D39 AL-) were used. In addition, three clinical *S. pneumoniae* isolates from CCUG, of which one was non-encapsulated, as well as five clinical AOM isolates from study III were utilized. Seven oral viridans *Streptococcus* strains from different culture collections were also used for comparisons of cytokine inducing capacities. The *S. pneumoniae* strains were cultured in broth to late log phase, with or without the autolysin inhibitor choline chloride. After washing, the bacteria were adjusted to the same concentrations and UV-inactivated, which was confirmed by negative viable count. At least 80% of pneumococci allowed to autolyse were fragmented, while the majority of autolysis-inhibited bacteria remained intact.

Preparation and *in vitro* stimulation of human blood mononuclear cells (I, II, III)

The capacity of different bacteria to induce cytokine production *in vitro* was studied on peripheral blood mononuclear cells (PBMC), according to a slightly modified protocol used earlier at the laboratory (198). The cells were prepared from blood-donor buffy coats by density gradient centrifugation, washed and adjusted to

the same concentration. The PBMC were incubated with UV-inactivated bacteria of different defined concentrations for 24 hours or 5 days.

Assessment of inflammatory mediators (I, II, III, IV)

Cytokines were measured in the supernatants after bacterial stimulation of PBMC for 24 h (IL-1β, TNF, IL-6, IL-8, IL-10, IL-12) or 5 days (IFN-γ) by in-house ELISAs, extensively used by several researchers at our and neighbouring departments (261). The levels of PGE_2 were measured after 24 h of incubation by using a commercial Enzyme Immunoassay (EIA) system.

Phagocytosis assay (I, II)

To measure phagocytosis, PBMC, prepared from blood-donor buffy coats by density gradient centrifugation, were incubated with bacteria for 30 min. A few Gram-positive species (*L. casei*, *L. monocytogenes* and *S. salivarious*) were incubated with PBMC for 16 h as well. After washing, the cells were spun onto glass slides, stained and examined in the microscope. The average number of surface-associated or internalized bacteria per monocyte was determined by counting at least 40 monocytes in the microscope.

Patients with acute otitis media (AOM) (III)

47 children visiting an ear, nose and throat clinic (Lundby Hospital, Gothenburg, Sweden) between January 2004 and May 2005 due to spontaneous perforated AOM were included in the study. The ages of the children ranged from 4 months to 14 years, but the median age was just over two years (Table 2). The perforation of the tympanic membrane occurred no more than 24 h before clinical examination was performed. Patients with chronic otitis media or tympanostomy tubes were excluded. During clinical examination, the fluid in the ear canal was sucked up in a container and the tube was flushed with sterile NaCl. Culture was performed bedside, while the remaining middle ear fluid was freezed for later assessment of inflammatory mediators and detection of microbes by PCR (see below). The study was approved by the Ethics Committee at the University of Gothenburg, Sweden.

Patients with secretory otitis media (SOM) and controls (IV)

60 children who were scheduled for surgery with insertion of tympanostomy tubes due to secretory otitis media (SOM) were included in this pilot study (Table 2). The principal aim of the double-blind, placebo-controlled study was to investigate the effects of nasal spray treatment with viridans streptococci or lactobacilli on SOM. The children were between one and eight years old (median 32 months) and had been followed clinically at the Lundby ear, nose and throat clinic in Gothenburg for at least two (median 6) months due to SOM. Exclusion criteria were severe underlying disease, immune deficiency, valvular heart disease or congenital heart defects, sore mucosa, antibiotic treatment within the last month, or upper

respiratory tract infection in the week preceding recruitment. Nineteen children (1-6 years of age, median 40 months) without infection or middle ear disease, operated on for other reasons (mostly ankyloglossia) served as non-treated controls. The study was approved by the Ethics committee at the University of Gothenburg.

Table 2: Patients recruited in the AOM and SOM studies, respectively.

	AOM	SOM			
Treatment	-	*S. sanguinis*	*L. rhamnosus*	Placebo	Controls
Age (months, median)	26	36	31	32	38
No. included	47	20	20	20	19
No. excluded	0	1	2	3	-
Remaining for evaluation	47	19	18	17	19
MEF collected	47	12	12	11	-

Study design SOM probiotic study (IV)

The overall study design is showed in Figure 8.

Day 0:

Clinical examination by otomicroscopy was performed at inclusion. A nasopharyngeal sample was obtained by a swab for assessment of bacterial flora and inflammatory mediators. After culture, the nasopharyngeal fluid was kept in freeze for later analysis of inflammatory mediators. The patients were randomized for treatment with *S. sanguinis*, *L. rhamnosus* or placebo for 10 days.

Day 10:

The effects of the probiotic spray were evaluated by otomicroscopy after 10 days of treatment. All clinical examinations were performed by the same ear, nose and throat specialist, who was blinded to the treatment of the patients. The clinical outcome was defined as “no improvement” (no change in fluid level), “some improvement” (slightly less fluid than at inclusion in at least one ear), “much better” (significant less fluid and more air in the middle ear) or “cured” (no fluid seen through the tympanic membrane). After clinical examination, the patients were operated on with insertion of a tube into the tympanic membrane under general anaesthesia. During surgery middle ear fluid was collected, and a nasopharyngeal swab was taken for bedside bacterial culture and later assessment of inflammatory mediators. Surgery was cancelled if the fluid had disappeared or almost disappeared and the patient had no signs of negative pressure in the middle ear. Acute infection presenting an anaesthetic risk was also a reason for cancelled

surgery. In those children not operated upon, a nasopharyngeal sample was taken. A nasopharyngeal swab was also used for bacterial culture and measurement of inflammatory mediators in non-treated control children.

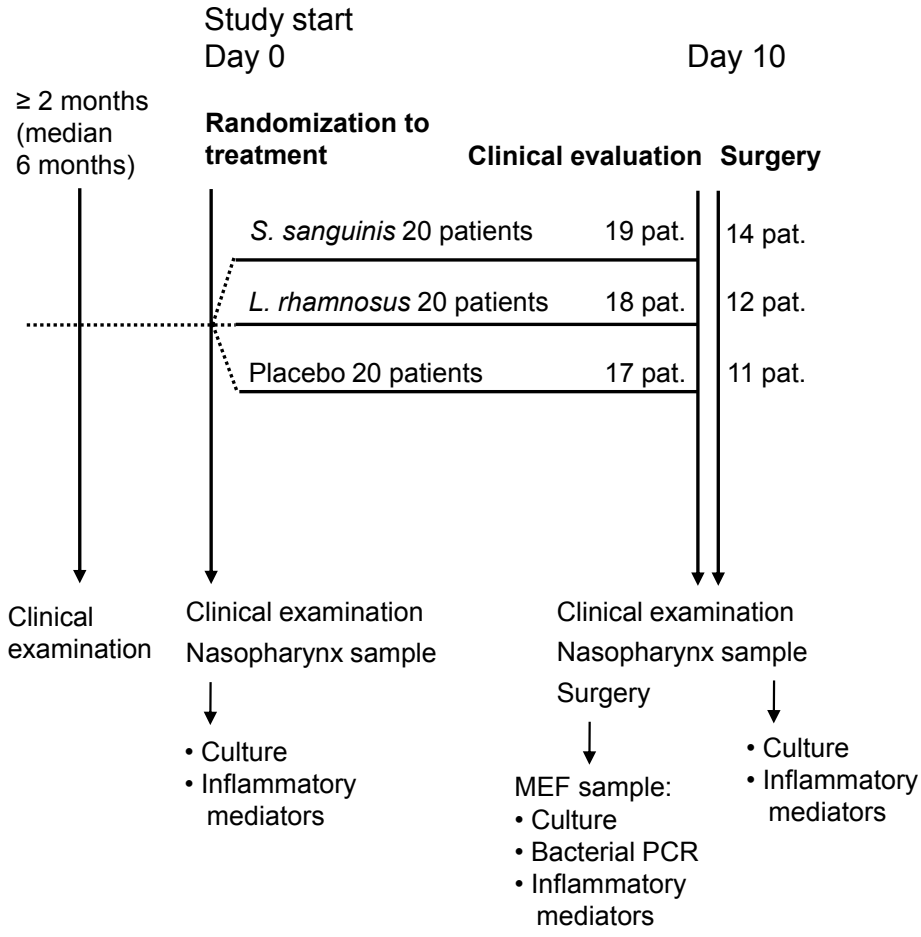

Figure 8: Study design of the SOM study.

Detection of microbes (III, IV)

Culture

Semi-quantitative culture was performed bedside on diluted nasopharyngeal secretions and middle ear fluids, respectively, from SOM patients, and from diluted exsudates obtained from patients with spontaneous perforated AOM. A calibated loop was streaked on suitable agar plates, which were transported within 4 hours to the laboratory and incubated under optimal conditions. *S. pneumoniae*, *H. influenzae*, *M. catarrhalis*, Group A streptococci, viridans streptococci (study IV) and lactobacilli (study IV) were enumerated and identified using standard bacteriological methods.

Bacterial PCR

Middle ear fluids from AOM and SOM patients were analysed by PCR for detection of *S. pneumonae*, *H. influenzae* and *Moraxella* spp. The extraction and preparation of DNA was performed by using a commercial preparation kit, with minor modifications for reliable digestion of the thick Gram-positive cell wall as described elsewhere (262, 263). A duplex PCR assay was performed for simultaneous detection of *S. pneumoniae* and *Moraxella* spp., and another PCR assay for detection of *H. influenzae*. The primers were previously published as well as the protocol, which we used with minor modifications (248). The sensitivity was found to be approximately 10 bacterial cells, and the PCRs were considered specific for *S. pneumoniae* and *H. influenzae*, while other *Moraxella* spp. than *M. catarrhalis* could give positive results.

Viral PCR

Twenty-five MEFs from the AOM patients were analysed by multiplex real-time PCR at the Viral Laboratory, Sahlgrenska University Hospital, Gothenburg, for the presence of rhinovirus, coronavirus, influenza A and B, RSV, adenovirus, metapneumovirus and enterovirus (264).

Statistical analyses (I, II, III, IV)

The overall cytokine pattern induced by 37 different bacterial species in paper I was visualized by Principal component analysis (PCA) using the SIMCA-P software (Umetrics, Umeå, Sweden).

Further, Mann-Whitney test was performed for statistical analyses of the differences in cytokine responses to the Gram-positive and Gram-negative bacteria used in paper I. The cytokine response of each donor to each of the 14 Gram-positive strains was averaged, and compared to the average response to the 23 Gram-negative species. Mann-Whitney test was also used for comparison of the inflammatory mediator levels between groups in the AOM study (paper III). Moreover, the bacterial numbers and levels of inflammatory mediators were analysed by this statistical method in the SOM study (paper IV).

Wilcoxon matched pairs test was used in paper II for comparisons of cytokine responses in the experiments with autolysin-positive and autolysin-negative bacteria. The paired test was also used in paper IV for comparisons of bacteria and cytokines in nasopharynx before and after probiotic spray treatment.

Fisher´s exact test was used in the SOM study (paper IV) for statistical analysis of the clinical outcome in patients treated with viridans streptococci, lactobacilli or placebo. Fisher´s exact test was also used in this thesis to compare the bacterial findings either detected by culture or PCR in middle ear fluids from SOM patients with the bacterial findings in fluids from AOM patients.

RESULTS

Cytokine production and taxonomic relatedness (Paper I)

14 Gram-positive and 23 Gram-negative bacterial strains from five different phyla of medical interest were UV-inactivated and incubated with peripheral blood mononuclear cells (PBMC) isolated from six blood donors. The levels of IL-12, IL-1β, TNF, IL-6, IL-8 and IL-10 were measured in the supernatants after 24 h, whereas IFN-γ was measured after 5 days of incubation. The results were analysed by Principal component analysis (PCA) to show the overall cytokine response pattern. In short, the PCA software performs a multivariate analysis of the data and calculates new synthetic variables (principal components), which contains as much variance as possible of that present in the material. The first principal component, which represents most of the variance, is represented by the horizontal axis and the second principal component by the vertical axis. Each of these dimensions is a composite of several variables. The contribution of the original variables to these synthetic variables appears in the loading plot (see below). It is, thus, possible to discern which of the original variables that contributes to the separation of the observations and which do not.

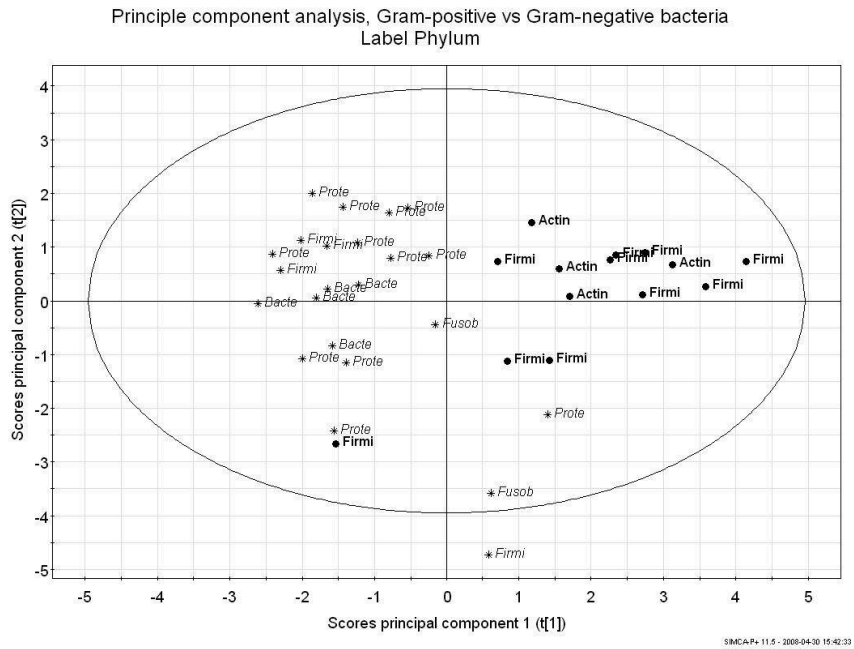

Figure 9

Figure 9 shows the position of the 37 bacterial species with respect to the composite cytokine pattern induced in the blood donors. As seen in the figure, the bacteria form two distinct groups separated along the horizontal axis. Bacteria belonging to the *Actinobacteria* and the *Firmicutes* phyla are positioned to the right, while bacteria belonging to the phyla *Proteobacteria*, *Bacteroidetes*, *Fusobacteria* as well as *Firmicutes*, are positioned to the left. Note, however, that bacteria in the *Firmicutes* phylum are found both in the left and the right group. The *Bacteroidetes*, *Fusobacteria* and *Proteobacteria* phyla contain only Gram-negative bacteria, while the *Actinobacteria* phylum contain only Gram-positives. The *Firmicutes* phylum contains both Gram-positive and Gram-negative bacteria.

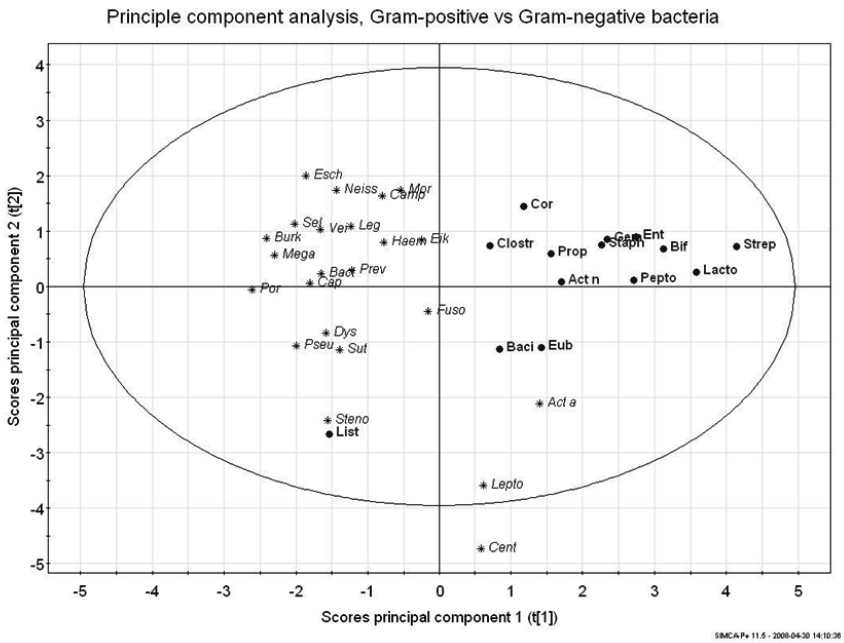

Figure 10

Figure 10 shows the same PCA pattern, but here the individual bacterial species and their Gram-staining characteristic is shown; Gram-positive bacteria are marked with dots and Gram-negative bacteria with stars, respectively. A striking pattern emerges: practically all bacteria positioned to the right are Gram-positive, while the left group contains mainly Gram-negative bacteria. Hence, Gram-negative bacteria belonging to the *Firmicutes* phylum are positioned together with other Gram-negative bacteria appearing to the left, while most Gram-positives in the *Firmicutes* phylum are positioned to the right in the Gram-positive group.

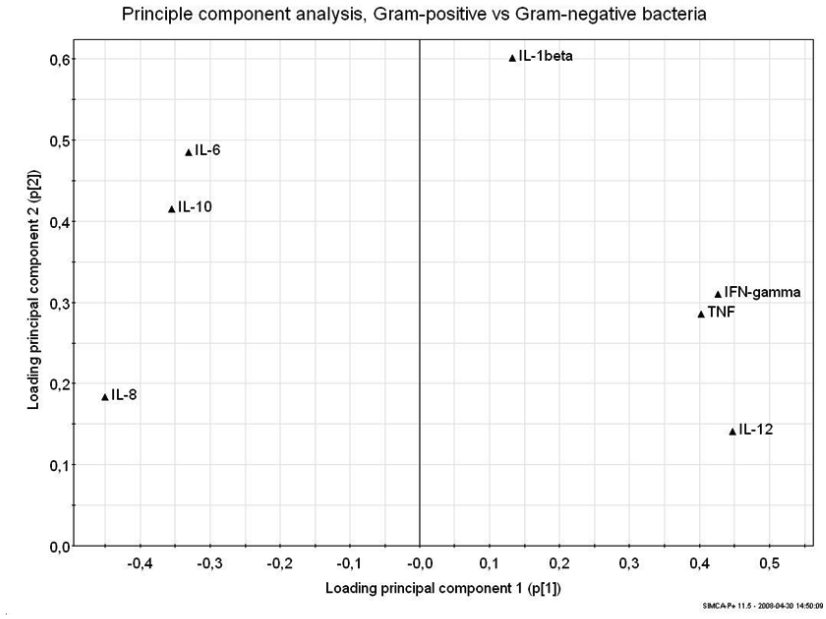

Figure 11

Figure 11 shows the variables (loadings) contributing to the position of the bacteria in the PCA representation. IL-12, TNF and IFN-γ are positioned to the right, while IL-6, IL-8 and IL-10 are appearing to the left. Thus, the Gram-positive bacteria obtain their position to the right in the diagram due to their strong tendency to induce IL-12, TNF and IFN-γ in PBMC, and their comparatively weak tendency to induce IL-6, IL-8 and IL-10, while the Gram-negative bacteria are positioned to the left because they induce much IL-6, IL-8 and IL-10, but comparatively little IL-12, TNF and IFN-γ. The further to the right a bacterium appears, the most pronounced is its tendency to induce "the Gram-positive pattern of cytokines", and vice vesa for the Gram-negatives. Further, from the loading plot it is apparent that TNF and IFN-γ contains almost the same information, at least regarding the first two principal components, which carry the absolute majority of information. This means that they are highly correlated and a bacterium which induces much TNF also induces IFN-γ. This is quite interestingly, as we measure TNF after 24h, which means that it is, in practice, monocyte-derived. IFN-γ, on the other hand is produced by T (and NK) cells and is measured after 5 days´ culture. Interestingly, IL-1β is separated from the other cytokines. Hardly any relation exists between the ability of bacteria to induce IL-1β and their Gram-staining pattern.

When analysing the cytokine levels induced by the 37 different bacteria, the Gram-positive bacteria induced on average nine times more IL-12, seven times more IFN-γ, three times more TNF and 1.5 times more IL-1β than did the Gram-negative

bacteria. In contrast, the Gram-negatives induced three times more IL-10, twice as much IL-8 and 1.5 times more IL-6 than did the Gram-positive bacteria.

Thus, irrespective of taxonomic position, Gram-positive bacteria induce more IL-12, TNF and IFN-γ than do Gram-negatives, which instead induce more IL-6, IL-8 and IL-10 than do Gram-positives. The capacity to induce IL-1β is unrelated to the capacity to induce either the "Gram-positive" or the "Gram-negative" pattern.

Exceptions to the general cytokine pattern (Paper I and II)

As shown in Figure 10, some bacteria diverge from the general Gram-positive and Gram-negative pattern. The Gram-negative bacteria *Centipeda periodontii*, *Leptotrichia buccalis* and *Aggregatibacter actinomycetemcomitans* appeared separate from the Gram-negative group. They were all poor inducers of IL-6, IL-8 and IL-10 relative to other Gram-negative bacteria (Figure 1b in Paper I). Thus, their position in the PCA pattern was caused by this deviation.

Another notable exception to the general cytokine pattern induced by Gram-positive and Gram-negative bacteria was *Listeria monocytogenes*, which separated distinctly from the other Gram-positive bacteria in the PCA analysis (Figure 10). *L. monocytogenes* was found to be a very poor inducer of IL-12, IFN-γ, TNF as well as IL-1β, while the levels of IL-6, IL-8 and IL-10 were similar to those induced by other Gram-positive bacteria (Figure 1a in Paper I). As this was an interesting finding, we decided to investigate the inducing capacity of seven other *L. monocytogenes* strains belonging to different serovars and with various origins. Indeed, all *Listeria* strains that were tested were poor inducers of IL-12, IFN-γ, TNF and IL-1β while significant amounts of IL-6, IL-8 and IL-10 were produced (Table 2, Paper I). Furthermore, no relation between cytokine pattern and serovar or origin was seen.

IL-12 stimulates production of IFN-γ, which synergizes with TNF to activate bactericidal functions of phagocytes. Thus, IL-12, IFN-γ and TNF can be referred to as phagocyte-activating cytokines. *Streptococcus salivarius* is in Figure 10 positioned furthest to the right and was found to be a strong inducer of phagocyte-activating cytokines (Figure 1a in Paper I). The same pattern was seen when other viridans streptococci were tested (Figure 1, Paper II). However, the virulent *Streptococcus pneumoniae*, which is genetically very closely related to viridans streptococci, was an exceptionally weak inducer of the phagocyte-activating cytokines IL-12, IFN-γ and TNF. In contrast, the levels of IL-6, IL-8 and IL-10 were similar to the levels obtained by the other streptococci.

Thus, in contrast to other Gram-positive bacteria, L. monocytogens and pneumococci are exceptionally poor inducers of phagocyte-activating cytokines. Both these bacteria are recognized pathogens.

Pneumococci and autolysis (Paper II)

Pneumococci have a tendency to undergo autolysis when reaching the stationary phase of growth. This is mediated by enzymes called autolysins, which degrades the peptidoglycan layer making up the bulk of the cell wall of Gram-positive bacteria. The major autolysin in *S. pneumoniae* is called LytA. Since earlier studies have shown that soluble bacterial cell wall components induce much less phagocyte-activating cytokines than do intact Gram-positive bacteria (199, 200), we hypothesized that autolysis may be responsible for the different cytokine pattern obtained by pneumococci compared to other Gram-positive bacteria. To compare the cytokine inducing capacity of pneumococci capable of autolysing with that of intact pneumococci, seven clinical strains were grown in medium with or without excess choline. Growth in high concentrations of choline is known to inhibit the function of the pneumococcal autolysins (265). PBMC were incubated with intact and fragmented bacteria, respectively, and cytokines were measured in the supernatant. As shown in Figure 2B Paper II, intact pneumococci induced significantly more IL-12, TNF and IFN-γ than did fragmented pneumococci, while similar (or higher) levels of IL-6, IL-8 and IL-10 were induced by intact and fragmented bacteria. Similar results were seen when a mutant *S. pneumoniae* strain, prevented from undergoing autolysis by mutation in the LytA-gene, was compared with the isogenic wild-type bacteria (Figure 2C Paper II).

During autolysis the intracellular toxin pneumolysin is released from the bacteria and has toxic properties on cells (266). To exclude that pneumolysin was responsible for the low production of phagocyte-activating cytokines in response to autolysed pneumococci, a pneumolysin-mutant strain was compared with a wild-type strain, both of which can autolyse. The cytokine pattern induced by these strains was practically identical, which excludes pneumolysin as the cause of blunted production of phagocyte-activating cytokines.

Intact pneumococci were admixed with graded concentrations of the corresponding autolyzed bacteria and the mixture was incubated with PBMC. The results are shown in Figure 3, Paper II. Interestingly, the production of IL-12, TNF and IFN-γ in response to intact bacteria was inhibited in a dose-depended manner when fragmented bacteria were added. In contrast, the amounts of IL-6, IL-8 and IL-10 were unaffacted or increased when autolysed pneumococci were added. Practically the same results were obtained when the mutant *S. pneumoniae* strain, incapable of undergoing autolysis, was admixed with various concentrations of autolyzed wild-type bacteria.

Thus, the presence of autolyzed pneumococci reduces the production of phagocyte-activating cytokines (IL-12, TNF and IFN-γ) in response to intact bacteria.

Phagocytosis assay (Paper I,II)

The cytokine response to different bacteria was studied in relation to phagocytosis of that particular strain. After incubation with UV-inactivated bacteria for 30 min, PBMC were spun onto microscope slides, stained and examined in the microscope. More Gram-positive bacteria than Gram-negative bacteria were seen internalized at this time-point, but the difference was not significant ($p=0.32$), and both Gram-negative and Gram-positive species were among those bacteria that were most readily phagocytosed. Further, the cytokine pattern induced by individual strains was unrelated to the degree of phagocytosis of the strain in question.

Some bacteria were only bound to the monocytic surface, but not internalized by 30 min. In these cases, we investigated phagocytosis also after 16 h incubation with PBMC. The rate of phagocytosis was highly increased for the Gram-positive bacteria *S. salivarius* and *L. casei*, while few *L. monocytogenes* were internalized even at this time-point. However, as many monocytes were in bad condition after 16 h, this time-point could not reliably be used for assessment of phagocytosis.

We also examined the consequences of autolysis for phagocytosis of *S. pneumoniae*. Wild-type pneumococci were poorly phagocytosed, which is a well-known phenomenon related to their thick capsule. After 30 min incubation with PBMC, no pneumococci were seen inside the monocytes. However, intact pneumococci that were prevented from autolysis, bound in chains on the monocyte surface. In contrast, wild-type pneumococci that could autolyse rarely interacted with the cells. Futher, when equal amounts of intact and autolysed pneumococci were added to the monocytes, the surface interaction of intact bacteria was significantly reduced compared to the results obtained by interaction with intact pneumococci alone. To better study the effects of autolysis on phagocytosis, we used a capsule-negative *S. pneumoniae* strain, which was cultured in excess choline to inhibit autolysis. In contrast to the encapsulated strains, capsule-negative intact pneumococci were readily ingested by monocytes. When this strain was allowed to autolyse, however, phagocytosis was significantly reduced. The same inhibition of phagocytosis was seen when encapsuled, autolyzed pneumococci were admixed with intact, capsule-negative pneumococci.

Thus, Gram-positive and Gram-negative bacteria are, in general, phagocytosed to the same degree. Wild-type pneumococci and L. monocytogenes were very poorly phagocytosed. Moreover, fragmented pneumococci appear to block access of intact bacteria to the monocyte surface and phagocytosis.

Microbial findings in middle ear fluids from patients with spontaneous perforated acute otitis media and secretory otitis media (Paper III and IV)

Middle ear fluid was collected from 47 children with spontaneous perforated tympanic membrane due to acute otitis media (AOM). Middle ear fluids were also collected from 35 children who were operated on for insertion of tympanic tubes due to longstanding middle ear effsuin in secretory otitis media (SOM). These children participated in a pilot study investigating treatment with probiotic nasal spray to resolve the long-standing exsudate (see below). Diluted middle ear fluid was cultured bedside and thereafter frozen for later analysis of microbe nucleotides and inflammatory mediators.

The bacterial pathogens found in the exsudates are shown in Table 3. In AOM, culture was positive in 47%, and PCR only little improved detection rates. Thus, a surprisingly high proportion of samples were negative regarding bacteria by both methods. *S. pneumoniae* and *H. influenzae* were the most common pathogens detected by culture, 23% and 21%, respectively (one sample contained both *S. pneumoniae* and *H. influenzae*). All culture-positive samples were also positive by PCR except one sample containing pneumococci. In addition, group A streptococci was found by culture in one single sample, whereas PCR was not designed to detect this pathogen. In seven culture-positive samples, the PCR detected not only the bacteria found by culture in each sample, but also another one or two bacterial pathogens.

Table 3: Bacterial pathogens detected by culture or PCR in middle ear fluids from patients with acute otitis media (AOM) or secretory otitis media (SOM), respectively.

		Positive (%)	*S. p.* (%)	*H. i.* (%)	*M. c.* (%)	GAS (%)	>1 pathogen (%)
AOM	Culture (n=47)	22 (47)	10 (21)	9 (19)	1 (2.1)	1 (2.1)	1 (2.1)
	PCR (n=47)	24 (51)	6 (13)	6 (13)	2 (4.3)	N.a.	10 (21)
SOM	Culture (n=34)	12 (35)	2 (5.9)	5 (15)	4 (12)	0	1 (2.9)
	PCR (n=32)	24 (75)	2 (6.2)	6 (19)	9 (28)	N.a.	7 (22)

N.a.= not applicable, *S. p.*= *S. pneumoniae*, *H. i.* = *H. influenzae*, *M. c.* = *M. catarrhalis*, GAS = group A β-hemolytic streptococci

In SOM, middle ear effusions were culture-positive in 35%, while the detection rate increased to 75% by PCR (p=0.0015). Thus, significantly more SOM than AOM middle ear fluids were PCR positive for bacterial pathogens (p<0.05). Moreover, Gram-negative bacteria were more often detected in SOM than AOM middle ear fluids using PCR for detection (p<0.05). This difference in pathogen distribution between AOM and SOM effusions could not be shown when the bacteria were detected by culture (p=0.61).

Bacterial AOM is often preceded by a viral upper respiratory infection. Twenty-five exsudates from spontaneously perforated AOM were analysed by PCR for the presence of common upper respiratory viruses. Viral DNA was detected in 20% of the samples, namely rhinovirus (n=4) and coronavirus (n=1). These samples were also culture positive for *H. influenzae* and/or *S. pneumoniae*.

Thus, in acute otitis media, culture was negative in half of the cases and this was only marginally improved by PCR. In secretory otitis media, PCR detected a high rate of bacteria and Gram-negative pathogenic bacteria were more often detected than in acute otitis media.

Inflammatory mediators in relation to microbial findings in MEF

Inflammatory mediators in middle ear fluids from patients with spontaneous perforated AOM and longstanding SOM were measured and related to the presence of pathogens detected by culture and/or PCR.

As shown in Figure 12, the levels of IL-1β and IL-8 were significantly higher in effusions that contained live, cultureable bacteria than in culture-negative samples. This was true for both AOM and SOM. The most pronounced difference between culture-positive and culture-negative fluids was seen for IL-1β. In exsudates from AOM patients, IL-1β levels were 11 times higher in those exsudates from which bacteria could be cultured, compared to exsudates devoid of cultureable bacteria (median 110 vs <7.5 ng/ml), and the same pattern was seen in SOM (median 11 vs 1.8 ng/ml). TNF and IL-10 levels were also higher in culture-positive AOM samples compared to culture-negative. IL-10 was elevated in culture-positive SOM samples as well; however, there was no statistically significant difference between culture-positive and culture-negative samples. The presence of dead bacteria as represented by negative culture and positive PCR were associated with elevated cytokine levels in a few cases, but not in the majority (Figure 1, Paper III and Figure 3, Paper IV).

PGE_2 were found in equally high levels in both culture-positive and culture-negative AOM and SOM samples. The same results were obtained for IL-6 in AOM samples. In contrast, significant higher levels of IL-6 were shown in culture-positive SOM samples compared to negative samples (Figure 12).

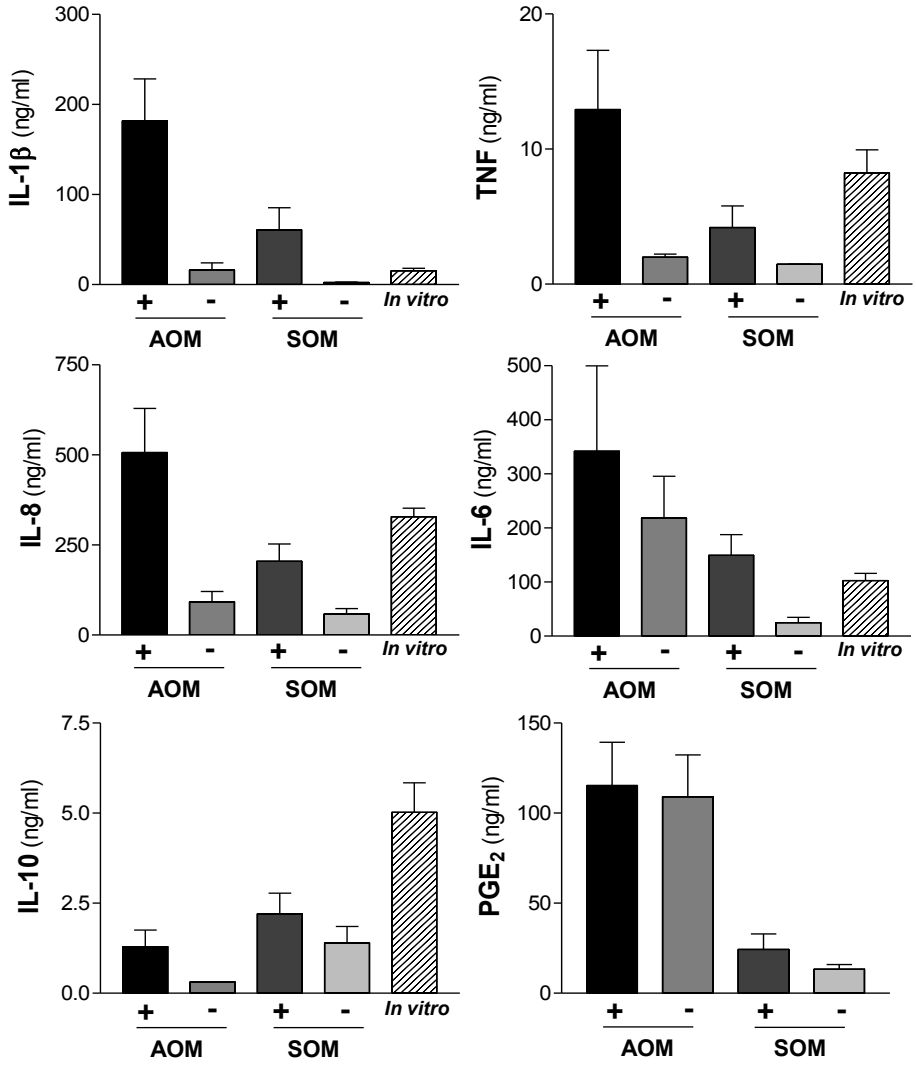

Figure 12: The levels of inflammatory mediators in culture-positive (+) and culture-negative (-) middle ear fluids from patients with acute otitis media (AOM) or secretory otitis media (SOM). Cytokines were also measured after in vitro stimulation of peripheral blood mononuclear cells (PBMC) with optimal concentrations of UV-inactivated AOM bacterial pathogens.

Inflammatory mediators in AOM and SOM samples

The duration of disease was short before sample collection in perforated AOM. Conversely, SOM had been ongoing for at least two months (median 6 months) before middle ear fluid was collected and analyzed. Furthermore, Gram-negative bacteria dominated in SOM. When comparing the levels of inflammatory mediators in culture-positive acute and secretory otitis exsudates, the levels of the pro-inflammatory cytokines IL-1β, TNF and IL-8 as well as PGE_2 were 2-3 times higher in AOM as compared to SOM ($p<0.05$) (Figure 12). In contrast, twice as much IL-10 was measured in SOM fluids compared to AOM exsudates ($p<0.05$). The levels of IL-6 were equally elevated in both diseases.

Inflammatory mediators in relation to microbes

As shown in Paper I and in earlier studies, Gram-positive bacteria induce higher levels of TNF *in vitro* compared to Gram-negatives, which instead induce more IL-6, IL-8, IL-10 and PGE_2 (200-202). To investigate if this pattern also were true for the cytokine pattern in otitis media, levels of inflammatory mediators in fluids containing *S. pneumoniae* or group A streptococci were compared with those in effusions containing *H. influenzae* or *M. catarrhalis*. However, no statistically significant differences in mediator levels between fluids containing Gram-positive or Gram-negative bacteria could be seen. This was true for both acute and secretory otitis media, and regardless of whether bacterial presence was determined by culture or PCR (data not shown). The presence or absence of virus did not influence the levels of inflammatory mediators in the middle ear fluids (data not shown).

Inflammatory mediators in AOM and SOM fluids compared to *in vitro* responses

We compared the cytokine levels from culture-positive middle ear fluids with those obtained by *in vitro* stimulation of PBMC with optimal doses of UV-inactivated *S. pneumoniae*, *H. influenzae* and *M. catarrhalis*. IL-6 and TNF could be induced in equally amounts *in vitro* as those seen in middle ear exsudates (Figure 12). In contrast, IL-1β and IL-8 levels were on average 11 and 3.5 times higher in AOM samples than could be induced *in vitro* ($p<0.01$). However, in SOM samples, the levels of IL-1β were equal to those obtained by *in vitro* stimulation, and IL-8 levels were actually lower in SOM samples compared to the levels induced *in vitro* ($p<0.5$). Both in AOM and SOM samples, the IL-10 levels were lower than could be produced *in vitro*, and the high *in vitro* results were mainly due to Gram-negative stimulation ($p<0.5$).

Thus, in general, cytokine levels were higher in culture-positive middle ear fluids compared to culture-negative fluids, and also higher in AOM than in SOM effusions. The cytokine levels were not related to the presence of Gram-positive or Gram-negative bacteria, respectively.

Bacteriotherapy in children with SOM (Paper IV)

Sixty children with longstanding SOM were randomised to nasal spray treatment with the Gram-positive bacteria *S. sanguinis* or *L. rhamnosus,* or placebo for 10 days prior to scheduled insertion of a plastic tube through the tympanic membrane. Clinical examination was performed at inclusion and after 10 days of treatment. Nasopharyngeal secretions were collected before and after treatment for analysis of bacteria and inflammatory mediators. Moreover, during surgery, middle ear fluid was collected for microbial and immunological analyses. Six patients were excluded due to AOM development or non-compliance and were not evaluated. Nineteen children operated for other reasons served as healthy controls.

Clinical effects of spray treatment

Clinical evaluation of the spray treatment was performed after 10 days of treatment by an experienced ear, nose and throat specialist, blinded regarding the treatment of the patient. The clinical examination included assessment of the amount of middle ear fluid as seen by otomicroscopy. In the *S. sanguinis* treatment group, 7/19 children were "much better" (i.e. had significantly less fluid and more air in the middle ear compared to base-line) or "cured" (no remaining fluid), compared to 1/17 patients in the placebo group (p=0.044) (Table 2, paper IV). Since another two patients showed "some improvement", 9/19 patients treated with *S. sanguinis* improved clinically compared to 3/17 in the placebo group (p=0.083).

Of the children treated with *L. rhamnosus*, three patients were "cured" or "much better" (p=0.60 compared with placebo) and another six patients showed "some improvement". Thus, 9/18 of the children in the *L. rhamnosus* treatment group were clinically improved (p=0.075 compared with placebo).

Bacterial flora in nasopharynx

A nasopharyngeal swab was obtained before and after 10 days of nasal spray treatment for culture of bacteria. In addition, nasopharyngeal culture was performed in 19 children without infection or middle ear pathology. There were no differences in the frequency of pathogens detected in the nasopharynx from children with SOM compared to healthy controls (Figure 2, Paper IV). However, the population numbers of *M. catarrhalis* were significantly higher in diseased children compared to controls (p=0.012), while healthy children tended to have more viridans streptococci (p=0.19). In none of the children treated with *S. sanguinis* could that particular strain be isolated from nasopharynx after treatment. In contrast, lactobacilli were found in a third of the patients treated with *L. rhamnosus*, but isolation of the probiotic strain was unrelated to clinical improvement. The presence of potential pathogenic bacteria (*S. pneumoniae*, *H. influenzae* and *M. catarrhalis*) in nasopharynx was not changed after spray treatment.

Bacterial pathogens in middle ear fluids in relation to spray treatment

Bacterial pathogens were detected in middle ear fluids by culture and PCR and the results were related to the spray treatment. Children treated with probiotic spray and placebo had very similar pathogen findings. However, clinical improvement seemed to be related to eradication of bacteria. Cultures from patients with complete or partial clinical improvement were positive for *S. pneumoniae*, *H. influenzae* or *M. catarrhalis* in 17% of the cases as compared with 45% of patients with no improvement (p=0.14). The results were similar by using PCR: 58% of the MEFs from children with improvement contained pathogens compared to 85% from children without improvement (p=0.12).

Inflammatory mediators in nasopharynx and middle ear fluids in relation to treatment

Inflammatory mediators were measured in nasopharyngeal secretions of healthy controls and from children with SOM before spray treatment. IL-8, IL-1β and PGE_2 could be detected in the nasopharyngeal fluid and the levels were similar in both groups. In the *L. rhamnosus* group, the levels of IL-8 increased significantly after treatment, and IL-1β and PGE_2 levels tended to increase as well. However, the levels of inflammatory mediators in nasopharynx were unrelated to clinical improvement.

Thus, nasal spray treatment with viridans streptococci seem to decrease middle ear fluid in children with SOM, but the mechanism remains to be elucidated.

DISCUSSION

Based on the structure of the cell wall, bacteria are divided into Gram-positive and Gram-negative bacteria. Gram-positive bacteria have a thick and sturdy cell wall, while the cell wall of the Gram-negatives is thin and surrounded by an outer membrane with LPS or LOS. Upon bacterial stimulation, monocytes and macrophages produce a wide range of inflammatory mediators. Earlier *in vitro* studies, using seven Gram-positive and Gram-negative species, have shown that mononuclear cells produce much more IL-12, TNF and IFN-γ in response to Gram-positive bacteria compared to Gram-negative bacteria (200, 202). Instead, more IL-6, IL-8 and IL-10 were produced in response to Gram-negative compared to Gram-positive bacteria (200, 202). These bacteria were derived from the *Actinobacteria*, *Firmicutes* and the *Proteobacteria* phyla. Whether bacteria from other phyla also induce cytokines according to this pattern is not known. Moreover, the bacterium *Veillonella parvula*, belonging to the "Gram-positive" phylum *Firmicutes*, induced considerable amounts of IL-12, although being Gram-negative (200). This prompted us to investigate the cytokine response to more bacteria belonging to the *Firmicutes* phylum as well as from other phyla of medical interest.

In total 37 Gram-positive and Gram-negative species from five different phyla were studied and the generalizability of the previous observed cytokine pattern was confirmed. We could also show that the tendency of Gram-positive and Gram-negative bacteria to induce different cytokine patterns crosses taxonomical borders. Hence, in general, Gram-positive bacteria from different phyla induce more IL-12, TNF and IFN-γ than Gram-negatives. Hardly any IL-12 was induced by the Gram-negative bacteria, including those belonging to the *Veillonellaceae* family in the *Firmicutes* phylum.

IL-12, TNF and IFN-γ may together be referred to as phagocyte-activating cytokines. IL-12, produced by monocytes/macrohages stimulates IFN-γ production and increases the cytotoxic activity of T cells and NK cells (185). IFN-γ, in turn, stimulates monocytes/macrohages to produce more IL-12 (the IL-12/ IFN-γ loop). IFN-γ also enhances bactericidal mechanisms in macrophages together with TNF (187). This cell mediated immunity has since long been regarded as critical in the defence against intracellular pathogens (192, 267, 268). However, the phagocyte-activating cytokines IL-12, TNF and IFN-γ also seem important in the host defence against extracellular Gram-positive bacteria, such as *S. aureus* (194), group B streptococci (195) and *S. pneumoniae* (196, 269-271). The enhance killing capacity of macrophages, provided by the phagocyte-activating cytokines, may be necessary for digestion of the thick and sturdy cell wall of Gram-positive bacteria, which may

contain up to 50 layers of peptidoglycan, compared to one or very few layers in Gram-negative bacteria.

Phagocytosis has also been shown to be crucial for the production of IL-12 in response to lactobacilli and bifidobacteria, respectively (199, 272). When phagocytosis was blocked by the action of cytochalasin, the IL-12 production was reduced in a dose-depended manner (199). In the absence of specific antibodies encapsulated pneumococci are resistant to phagocytosis, and the IL-12 levels measured in our study in response to intact pneumococci were also much lower than those induced by other Gram-positive bacteria. Instead of being phagocytosed, intact pneumococci were seen interacting with the monocyte surface. An intact non-encapsuled pneumococcal strain, which was readily phagocytosed, induced almost three times more IL-12 than an intact encapsuled pneumococcus strain, which strengthens the hypothesis that internalization of Gram-positive bacteria is necessary for their induction of IL-12.

Receptor activation and cytokine induction by intact bacteria may occur on the extracellullar surface of the macrophage, but also after phagocytosis inside the phagosome. TLR2 is believed to play an important role in the host defence against Gram-positive bacteria (146-148), and active recruitment of TLR2 to phagosomes containing microbes has been showed (137). Thus, our results suggest that the main trigger of IL-12 production is phagocytosed intact Gram-positive bacteria, localized to the phagosome. The receptors may be coupled to different signalling pathways when present in the phagosome membrane than when present in the cytoplasmatic membrane.

The immune response to some Gram-positive bacteria differed from the general pattern. *Listeria monocytogenes* and *Bacillus cereus* were particularily poor inducers of the phagocyte-activating cytokines IL-12, TNF and IFN-γ. Interestingly, in contrast to other Gram-positive bacteria, *B. cereus* and *L. monocytogenes* both contain *meso*-DAP instead of lysine in their peptidoglycan stem peptide (6). *Meso*-DAP containing peptidoglycan, present in Gram-negative bacteria, is sensed by the intracellular receptor NOD1, whose activation leads to transcription of inflammatory associated genes. In a recent study, activation of NOD1 was associated with chemokine production and neutrophil recruitment, whereas TNF, IL-12 and IFN-γ were not induced (273). However, if one supposes that production of IL-12 is induced by activation of receptors common for both Gram-positive and Gram-negative bacteria, stimulation of NOD1 by Gram-negative peptidoglycan could induce active inhibition of IL-12 production and thereby reduced cell mediated immunity. This could be beneficial for the intracellular pathogen *L. monocytogenes*. However, it has recently been shown that bacterial load and survival of *L. monocytogenes* was increased in NOD1 deficient mice (274).

L. monocytogenes is a food-borne pathogen capable of causing meningitis and sepsis in newborn, elderly and immunocompromised patients, as well as severe fetal infections in pregnant women. *L. monocytogenes* enters cells by phagocytosis or induced uptake in non-phagocytic cells, including enterocytes and hepatocytes (275). Replication is restricted to the cytosol, and the bacteria therefore need to escape from the phagosome after phagocytosis. This is mediated by the pore-forming toxin listeriolysin O, which is a major virulence factor of *L. monocytogenes* (276). Interestingly, listeriolysin O deficient bacteria fail to active caspase-1 and release of IL-1β via NALP3 inflammasome formation (277). In our assay, bacteria were UV-inactivated, and the *Listeria* strains used in our study should be functionally devoid of listeriolysin O. This is supported by the very poor capacity of *L. monocytogenes* to induce IL-1β production in our assay. Mice devoid of TLR2 have decreased ability to produce TNF and IFN-γ in response to *L. monocytogenes* (148). Moreover, production of TNF was completely abolished in mice macrophages lacking MyD88, suggesting a crucial role for this protein (148). CD14, known to be a co-receptor for TLR4, also seems to act together with TLR2 in the recognition of *L. monocytogenes* (278).

Various viridans streptococci were strong inducers of the phagocyte-activating cytokines IL-12, TNF and IFN-γ. However, *S. pneumoniae*, genetically related to *S. mitis*, induced remarkably low levels of these cytokines. The failure to induce IL-12 was shown to depend on the action of the pneumococcal autolysin. Autolysins are peptidoglycan hydrolysing enzymes which are involved in several fundamental steps of the bacterial life cycle, such as cell cell wall turnover, cell division and separation (279). Many Gram-positive bacteria possess autolysins, including *B. cereus* and *L. monocytogenes* (280, 281), which, coincidentally, also gave the lowest IL-12 response in our screening. The pneumococcal autolysins are shown to be associated with enhanced virulence of the bacteria in animal models (53-55), but the mechanism has not been elucidated. We demonstrated that pneumococci, allowed to autolyse induced significantly lower levels of IL-12, TNF and IFN-γ than intact bacteria. This inhibition was independent of pneumolysin, which refuses the proposed hypothesis that the virulence endowed by autolysin would be related to its capacity to set pneumolysin free. Moreover, when fragmented pneumococci were admixed with intact bacteria, we observed a dose-depended inhibition of the phagocyte-activating cytokines. These results agree with earlier findings that Gram-positive bacteria need to be intact to induce IL-12 production (199, 272), and that soluble components of the gram-positive the cell wall, such as peptidoglycan, MDP and LTA, do not induce any IL-12 (199, 200, 272) and even inhibit IL-12 production (199, 282).

We regard the capacity to generate fragments from decaying bacteria of low viability as an important virulence mechanism that may explain why pneumococci with the LytA gene are more virulent than strains without LytA.

The mechanism of this IL-12 inhibition is not known. One part of the explanation could be blocking of phagocytosis. We observed that pneumococci allowed to autolyse were internalized at a significantly lower rate than intact bacteria and that addition of fragmented bacteria blocked access of the intact pneumococci to the monocyte surface and phagocytosis of a non-encapsulated strain.

It has been reported that NOD2 could be involved in the negative regulation of IL-12 production by bacterial cell wall fragments (282, 283). The inhibition could also be mediated, at least in part, by stimulation of TLR2 with bacterial fragments. The inhibition of IL-12 production by peptidoglycan was mitigated in TLR2 deficient macrophages compared with the inhibition in wild-type macrophages (282). A proposed model for the regulation of IL-12 production is shown in Figure 13. We suggest that the strongest trigger of IL-12 is phagocytosed intact Gram-positive bacteria. Once the bacteria are killed there is no need for further activation of bactericidal functions in the macrophage, and hence the IL-12 production is turned off. Bacterial cell wall fragments, released after digestion of the bacteria, are sensed by intra- and/or extracellular receptors, but in contrast to intact bacteria, the fragments fail to crosslink receptors and/or bind to accessory proteins, which results in an inhibitory down-stream signal.

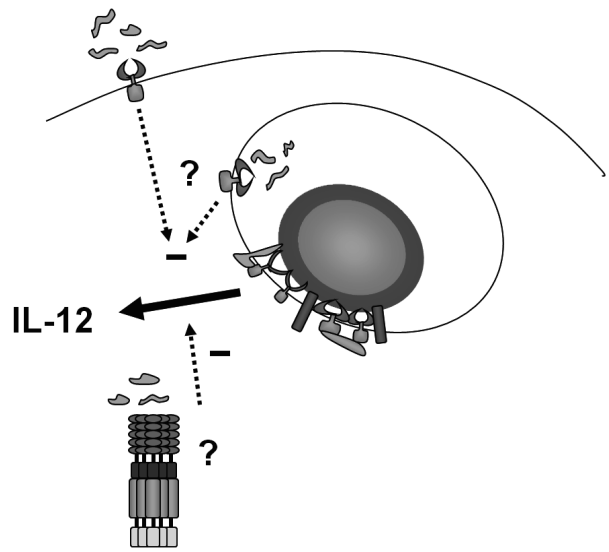

Figure 13: Proposed model for the production of IL-12 in response to intact Gram-positive bacteria. Phagocytosed bacteria may induce IL-12 by crosslinking several receptors and/or accessory proteins in the phagosome. Bacterial cell wall degradant products could also be sensed by one or several receptors, but this recognition leads to an inhibitory signal, perhaps due to abscence of receptor cross-linking.

Inhibition of cytokine production by bacterial fragments, as shown for the phagocyte-activating cytokines, has not been observed for IL-6, IL-8 or IL-10. Similar levels of IL-6 were produced in response to intact bifidobacteria as to bacteria pre-treated with bacterial fragments (199). In our experiments with autolysed pneumococci, similar or slightly increased levels of IL-6, IL-8 and IL-10 were produced when intact pneumococci were admixed with different concentrations of autolysed bacteria.

This thesis confirms the earlier observation that Gram-negative bacteria generally induce more IL-6, IL-8 and IL-10 from human monocytes than do Gram-positive bacteria, and shows that this tendency was seen regardless of taxonomic divisions. Gram-negative bacteria induced variable amounts of TNF and IFN-γ, although markedly lower levels than did most Gram-positive bacteria. The levels of IL-1β also varied considerably between different Gram-negative species, whereas IL-12 was practically not induced at all. Compared to Gram-positive bacteria, the Gram-negative cell wall is thin, and is readily digested by activated neutrophils. Therefore, there is no need for additional stimulation of the macrophage killing capacity by the IL-12/IFN-γ loop. Macrophage activation and IL-12 production is potently inhibited by IL-10 (190), which is produced in large amounts in response to Gram-negative bacteria.

The thin outer membrane is also susceptible to complement induced lysis, which causes leakage of the intracellular contents and death of the bacteria. The complement cascade is activated by direct binding of complement factors to the bacterial cell wall or by binding to antibodies associated with the bacterial surface. Important factors for killing Gram-negative bacteria is therefore activated neutrophils, complement factors and antibodies. Neutrophils are recruited to the infected tissue and activated by IL-8 (122, 123). Interaction of the neutrophils with endothelial cells is facilitated by vasodilatation, promoted by PGE_2 (125), and the expression of endothelial adhesins, promoted by IL-1β and TNF (118). Complement factors are mainly produced in the liver and the production is enhanced by the action of IL-6 (129). IL-6 and IL-10 also promote production of antibodies (130, 188). Thus, the cytokine profile induced by Gram-negative bacteria mainly leads to activation of humoral defences and neutrophil recruitment.

LPS, located in the outer membrane, is the most important virulence factor of Gram-negative bacteria. Interestingly, the levels of IL-6, IL-8, IL-10 and PGE_2 induced by intact Gram-negative bacteria could be mimicked by stimulating monocytes with LPS extracts (200-202). LPS is recognized indirectly by TLR4, whose activation leads to production of several inflammatory mediators (155).

The Gram-negative bacteria *Centipeda periodontii*, *Leptotrichia buccalis* and *Aggregatibacter actinomycetemcomitans* were separated from the other Gram-negatives in the PCA plot, and were also shown to be poor inducers of IL-6, IL-8 and IL-10 relative to other Gram-negative bacteria. *C. periodontii* is a bacterium

belonging to the normal oral flora. It has been detected in endodontic infections, but there was no relation to clinical symptoms, and the contribution of the bacterium to disease is uncertain (284). The LPS of *C. periodontii* has been isolated and characterized, and was found to induce strong immunobiological activities in mice (285). *L. buccalis* also belongs to the normal oral flora and seldom causes infections. It may cause invasive disease, but primarily in neutropenic patients. The LPS of *L. buccalis* is much less potent than that of *Escherichia coli*, which might explain the low pathogenecity of this species (286). *A. actinomycetemcomitans* is present in the normal oral flora as well, but is also associated with endocarditis, soft tissue infections and periodontitis. Leukotoxin is one known virulence factor that may contribute to disease (287). Thus, the ability to cause human disease differs between *C. periodontii*, *L. buccalis* and *A. actinomycetemcomitans*, and other factors besides the capacity to induce cytokines are most likely important in virulence.

When 37 bacterial species were tested for the capacity to induce IL-1β in mononuclear cells, slightly higher levels were obtained by Gram-positive bacteria compared to Gram-negative. However, the variation in cytokine levels between species was large, and PCA analysis revealed that the capacity of strains to induce IL-1β was unrelated to the capacity to induce any other of the investigated cytokines. The way IL-1β is produced also differs from the other cytokines investigated in this study. Production of active IL-1β requires two separate signals: one that initiates production of pro-IL-1β, and another that results in cleavage of pro-IL-1β and subsequent secretion of active IL-1β from the cell (116). The first signal is provided by microbes that stimulate TLRs and/or NODs leading to activation of NF-κB and MAPKs and production of pro-IL-1β. The other signal may also be derived from microbes, but can also be an endogenous danger signal, provided by stressed or necrotic cells. Activation by micobial or host danger signals leads to formation of an inflammasome, a multiprotein complex, which recruits and activates caspase-1. Activated caspase-1 cleaves pro-IL-1β to IL-1β which is then secreted out of the cell by yet unknown mechansisms.

Exceptionally high levels of IL-1β were measured in culture-positive middle ear fluids collected from children with spontaneously perforated acute otitis media (AOM). These extremely elevated levels were also demonstrated when the cytokine levels measured in middle ear effusions were compared to those obtained by *in vitro* stimulation of human monocytes with otitis media pathogens. Perforation of the tympanic membrane is associated with high degree of tissue damage and necrosis of host cells. The high IL-1β levels could be the result of inflammasome formation due to the activation by danger signals released from stressed and damaged cells in the infected middle ear mucosa in general, and from the perforated tympanic membrane in particular. However, perforation of the tympanic membrane may also be the consequence of high IL-1β levels in the middle ear fluid. IL-1β induces production of metalloproteases from fibroblasts and other

tissue cells (288). The tympanic membrane is composed of a cuboidal epithelium covering dense collagen-rich connective tissue, and metalloproteases are enzymes capable of digesting collagen and other matrix proteins (135).

Beside IL-1β, the inflammatory mediators TNF, IL-6, IL-8, IL-10 and PGE_2 were measured in exsudates from children with spontaneously perforated AOM. In addition, middle ear fluids were collected from children with secretory otitis media (SOM), participating in a study investigating the effects of probiotic treatment against the longstanding middle ear exsudates. Several mediators, especially IL-1β and IL-8, were markedly elevated in effusions containing live, culturable bacteria, as compared to culture-negative samples. IL-1β has previously been shown to be higher in culture-positive than culture-negative middle ear fluids from patients with AOM and SOM (228, 289), and the same has been shown for TNF and IL-8 in AOM patients (229, 230). We also showed that this was true in culture-positive AOM samples compared to negative, and IL-6 levels in culture-positive than culture-negative SOM samples.

IL-1β levels were 11 times higher in samples containing live bacteria compared to negative fluids. This exceptionally large difference in IL-1β levels has not been shown elsewhere. We assume that all cases of AOM had a bacterial cause, but that bacteria had been eliminated in the cases where the middle ear fluids were negative. Either the IL-1β levels had been equally high in the culture-negative fluids as long as they contained live bacteria, but elimination of bacteria was followed by a rather rapid reduction of cytokine levels. Proteases from neutrophils or metalloproteases might be able to reduce the levels of cytokines. Alternatively, the culture-negative fluids represented cases of otitis media caused by pneumococci that were autolyzed and, hence, escaped detection. It is well known in clinical bacteriological practice, that pneumococci are notoriously difficult to culture, due to their tendency to undergo autolysis. In this case, the presence of pneumococcal fragments during infection could counteract production of pro-inflammatory cytokines. The inhibition of TNF by this mechanism is discussed above, as shown in the pneumococcal autolysin study. Preliminary data also show that pneumococci are poor inducers of IL-1β compared to related viridans streptococci, suggesting that the production of IL-1β might be inhibited in the same way. Reduction of IL-8 could be a consequence of reduced IL-1β and/or TNF, as epithelial cells produce IL-8 in response to IL-1β and TNF (123).

IL-6 levels were elevated in AOM effusions with no detectable bacteria and in fluids with cultureable bacteria. Whereas acute inflammation is accompanied by gross neutrophil influx, SOM effusions are dominated by macrophages (104). This transition from a neutrophil to a mononuclear cell population is mediated by IL-6 (130). IL-6 inhibits production of IL-1β and TNF, favours the resolution of the neutrophil infiltrate and directs the transition from innate to acquired immunity (130, 131). Fragments from eliminated bacteria could perhaps stimulate continued

IL-6 production. The capacity of fragments to induce IL-6 has been showed by us in this thesis, as well as in earlier work (199). IL-6, produced in response to live bacteria before they were killed, might also remain in the middle ear fluid for a longer period than the other cytokines. Earlier work on a rat pneumococcal AOM model suggests a slow elimination of IL-6: While the production of IL-6 ceased within 24 hours after initiation of the infection, the cytokine was observed in the middle ear tissue at least five days later (290).

When further comparison between the levels of inflammatory mediators in AOM versus SOM effusions was performed, it was shown that IL-1β, TNF, IL-8 and PGE_2 levels were 2-3 times higher in AOM compared to SOM. These mediators are all central for recruitment of neutrophils to the site of infection during the early phase of infection. In contrast, twice as much IL-10 was measured in SOM compared to AOM fluids. This might be a result of the Gram-negative bacterial dominance seen in SOM. IL-10 inhibits cell mediated immunity, by down-regulating macrophage activation and production of IL-12 and IFN-γ (190). Perhaps this down-regulation is disadvantageous for the resolution of SOM. Thus, the differences in cytokine levels between AOM and SOM samples also reflect the characteristics of AOM as an acute inflammatory disease, compared to the more chronic condition of longstanding SOM.

When examining inflammatory mediators in ear infections to the findings of Gram-positive vs Gram-negative bacteria in the middle ear fluids by culture or PCR, no relation between the levels of inflammator mediators and Gram-staining pattern was seen. Though not shown in the papers, IL-12 and IFN-γ were also measured in the AOM and SOM samples. IL-12 could not be detected at all, while a few samples contained elevated IFN-γ levels. The Gram-negative bacteria detected in the exsudates were either *H. influenzae* or *M. catarrhalis*, while the Gram-positive bacteria were almost solely *S. pneumoniae* (group A streptococci were found in a single case). As we demonstrate in this thesis, pneumococci might be a bad choice for showing a superior capacity of Gram-positive bacteria to induce IL-12, TNF and IFN-γ compared to Gram-negative bacteria. If *S. pneumoniae* behave *in vivo*, as *in vitro*, autolysis of bacteria would generate bacterial fragments that efficiently inhibit the production of IL-12, TNF and IFN-γ, but can stimulate IL-6 production. Accordingly, serum levels of IL-6 have actually been showed to be higher in AOM caused by pneumococci than AOM casued by *H. influenzae* (291). In septic patients, especially those with late septic shock, the levels of TNF were significantly higher in patients with a pure Gram-negative infection, compared to those with a pure Gram-positive infection (292). Of note is that *S. pneumoniae* constituted a large fraction of the Gram-positive isolates in this study. Animal models also show that pneumococcal infection is not associated with elevated cytokine responses. For example, in an experimental rat meningitis model, no differences in the mRNA expression of IL-12, IFN-γ and IL-10 could be seen in the

brains of rats inoculated with either *H. influenzae* type B or pneumococci, and IL-6 and TNF mRNA levels were lower in *S. pneumoniae* inoculated rats (293).

Bacteria could only be cultured in about half of the cases in middle ear fluids from patients with spontaneous perforated AOM. This is a strikingly lower detection rate than earlier studies, which report >80% recovery rates in AOM with spontaneous perforation (226, 294). We could only slightly increase the detection rate of bacterial pathogens by using PCR. In contrast, PCR increased the detection rate to 75% in the SOM samples, compared to 35% by culture. This agrees precisely with earlier findings (247, 248). The difference in culture-positivity of AOM samples in our study compared to previous studies could perhaps be explained by differences in culture methods. However, even if the bacteria died during transport to the laboratory, their DNA should be readily detected by PCR.

The absence of bacterial pathogen DNA in the AOM specimens is quite remarkable. Could there be other bacteria not detected by our PCR that causes the infection? The bacterium *Alloiococcus otitidis* is frequently detected by PCR in AOM samples, but the clinical relevance is highly doubtful (295). Could viruses be responsible for causing the cases of AOM that were negative for bacteria? Viruses have been detected in high frequencies in middle ear fluids from AOM patients, especially by using PCR (107). The contribution of viruses to AOM development is generally accepted, although the high detection rates achieved by using PCR probably not reflect the clinical relevance of all these findings. When we analyzed our AOM samples with multiplex PCR, designed to detect a wide range of respiratory viruses, 20% were positive for either of two different viruses, namely rhinovirus and coronavirus. These samples were, however, also invariably culture positive for bacterial pathogens. Inflammatory mediators, such as IL-8 has been found to be higher in effusions containing both viruses and bacteria (107). However, we could not find any relations between inflammatory mediator levels and the presence or absence of viruses in the exsudates.

In our AOM study *S. pneumoniae* and *H. influenzae* were the most common pathogens detected by culture. *S. pneumoniae* has previously been isolated in most AOM patients with spontaneous rupture of the tympanic membrane (226, 294). Due to the severity of the AOM leading to rupture of the tympanic membrane, we believe that the infections in our study were caused mainly by *S. pneumoniae* as well. The absence of bacterial DNA could be due to an active elimination soon after killing of the bacteria. Perhaps DNases present in the middle ear effusion are involved in this process.

There was a Gram-negative dominance of bacteria detected by PCR in the fluids from SOM patients as compared to AOM. This distinction between AOM and SOM has been described earlier (25). *H. influenzae* is usually the most common species detected in SOM, both by culture and by PCR (25, 248). The high detection rate of bacteria by PCR in combination with the low isolation rate by culture

described by us and others (247, 248) highlights the hypothesis that bacteria reside in biofilms during SOM. Biofilms are highly organized networks with bacteria encased in matrix, usually localized to a surface (249). *H. influenzae* as well as *S. pneumoniae* and *M. catarrhalis* can form biofilms *in vitro* and *in vivo*, and biofilms have been demonstrated in children with SOM (252). Bacteria in biofilms have reduced growth rate and increased resistance to antibiotics and host defence. Thus, biofilms could be an important reason for the chronic characteristic of SOM and the low rate of culture-positivity.

SOM is characterized by persistent fluid in the middle ear without signs or symptoms of an acute infection. The condition is often a consequence of AOM, but may also develop spontaneously. The effusion in SOM often resolves spontaneously, but after a period of observation, the patients may be operated on with insertion of tympanostomy tubes during general anaesthesia. In a placebo-controlled double blind pilot study, we studied the effects of nasal probiotic spray treatment against the long-standing fluid in the middle ear. Sixty patients were treated with streptococci, lactobacilli or placebo for 10 days proceeding surgery.

In a study of AOM children, spray treatment with viridans streptococci not only decreased the recurrent rate of AOM infection, but also seemed to protect against SOM development (260). In the current study we used a *S. sanguinis* strain and a *L. rhamnosus* strain. Whereas the streptococcus strain has been tested in earlier spray treatment studies (258-260), the lactobacillus strain has primarily been used as a commercial supplement in dairy products. Both strains have shown good interfering capacity against *in vitro* growth of other microbes (data not shown). However, while *S. sanguinis* has shown excellent inhibiting ability against the middle ear pathogens *S. pneumoniae*, *H. influenzae*, *M. catarrhalis* and Group A streptococci, the lactobacillus strain has mainly been tested against intestinal pathogens such as *Salmonella* spp. and *E. coli*. Both *S. sanguinis* and *L. rhamnosus* are potent activators of the phagocyte-activating cytokines IL-12, TNF and IFN-γ, and also induce considerable amounts of IL-6, IL-8 and IL-10 (data not shown).

In this thesis, nasal spray treatment with *S. sanguinis* led to complete or almost complete resorption of the middle ear fluid in a third of treated patients with long-standing SOM, while only one patient out of 19 in the placebo group cured spontaneously. Treatment with lactobacilli was less effective.

We further investigated the mechanism for the beneficial effects of nasal spray treatment on clinical recovery. Due to the great inhibiting capacities of the *S. sanguinis* strain against *in vitro* growth of AOM pathogens, one could expect a change in the nasopharyngeal pathogenic flora. However, no change was observed in the flora in either of the treatment groups. Moreover, *S. sanguinis* was not isolated from nasopharynx in any case after spray treatment, suggesting that colonization of *S. sanguinis* was insignificant. Lactobacilli were isolated in a third of patients treated with *L. rhamnosus*, but their presence in nasopharynx after

treatment was not associated with clinical improvement. In contrast to other studies, we were not able to show any marked abnormalities, except for higher population levels of *M. catarrhalis*, in the nasopharyngeal flora of children with SOM compared to control children.

Another hypothesis was that *S. sanguinis* and *L. rhamnosus*, as potent activators of various cytokines, would stimulate innate immune responses to resolute the inflammation in the middle ear. For this purpose, inflammatory mediators were measured in nasopharynx and the middle ear fluid after spray treatment. However, *S. sanguinis*, which showed the most pronounced clinical effect on reducing the amount of middle ear fluid in SOM, induced no measurable effects on cytokine expression in the nasopharynx. In contrast, patients treated with *L. rhamnosus* had significantly higher levels of IL-8 in nasopharynx and tendencies of increased IL-1β and PGE_2 levels after treatment as well. However, no relation to clinical outcome was shown. No measurable levels of IL-12 and IFN-γ were seen (data not shown). Nor were there any differences in the levels of inflammatory mediators in middle ear fluids between the different treatment groups.

Investigation of bacteria and inflammatory mediators in the middle ear fluids might be the most relevant way to elucidate the mechanism of spray treatment on the reduction of the exsudate in SOM. However, in patients with clinical recovery there was no effusion to study. Perhaps an animal model is needed to elucidate the mechanisms behind the spray´s effects on SOM. However, we observed that the frequency of bacterial pathogens, foremost Gram-negative bacteria, tended to be higher in effusions from patients without clinical improvement. These pathogens might remain in the middle ear, continuous stimulating a low-grade inflammation, without being eliminated by the host defence.

This pilot study indicates that nasal spray treatment with viridans streptococci can reduce the amount of fluid in the middle ear of children with SOM. Thereby, probiotic spray treatment could postpone surgery or even rendering insertion of tympanostomy tubes unnecessary in many cases. As SOM is a serious problem for many patients, with hearing loss and recurrent surgery, nasal spray treatment with viridans streptotocci could be a relevant alternative in the treatment of SOM.

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REFERENCES

1. **Porter, J. R.** 1976. Antony van Leeuwenhoek: tercentenary of his discovery of bacteria. Bacteriol Rev 40:260-9.
2. **Whitman, W. B., D. C. Coleman, and W. J. Wiebe.** 1998. Prokaryotes: the unseen majority. Proc Natl Acad Sci U S A 95:6578-83.
3. **Loveland-Curtze, J., V. I. Miteva, and J. E. Brenchley.** 2009. Herminiimonas glaciei sp. nov., a novel ultramicrobacterium from 3042 m deep Greenland glacial ice. Int J Syst Evol Microbiol 59:1272-7.
4. **Jackson, C. R., H. W. Langner, J. Donahoe-Christiansen, W. P. Inskeep, and T. R. McDermott.** 2001. Molecular analysis of microbial community structure in an arsenite-oxidizing acidic thermal spring. Environ Microbiol 3:532-42.
5. **Popescu, A., and R. J. Doyle.** 1996. The Gram stain after more than a century. Biotech Histochem 71:145-51.
6. **Benko, S., D. J. Philpott, and S. E. Girardin.** 2008. The microbial and danger signals that activate Nod-like receptors. Cytokine 43:368-73.
7. **Baddiley, J., and A. L. Davison.** 1961. The occurrence and location of teichoic acids in lactobacilli. J Gen Microbiol 24:295-9.
8. **Coley, J., M. Duckworth, and J. Baddiley.** 1972. The occurrence of lipoteichoic acids in the membranes of gram-positive bacteria. J Gen Microbiol 73:587-91.
9. **Briese, T., and R. Hakenbeck.** 1985. Interaction of the pneumococcal amidase with lipoteichoic acid and choline. Eur J Biochem 146:417-27.
10. **Navarre, W. W., S. Daefler, and O. Schneewind.** 1996. Cell wall sorting of lipoproteins in Staphylococcus aureus. J Bacteriol 178:441-6.
11. **Howard, J. G., D. Rowley, and A. C. Wardlaw.** 1958. Investigations on the mechanism of stimulation of non-specific immunity by bacterial lipopolysaccharides. Immunology 1:181-203.
12. **Preston, A., R. E. Mandrell, B. W. Gibson, and M. A. Apicella.** 1996. The lipooligosaccharides of pathogenic gram-negative bacteria. Crit Rev Microbiol 22:139-80.
13. **Schweda, E. K., J. C. Richards, D. W. Hood, and E. R. Moxon.** 2007. Expression and structural diversity of the lipopolysaccharide of Haemophilus influenzae: implication in virulence. Int J Med Microbiol 297:297-306.
14. **Weiser, J. N., and N. Pan.** 1998. Adaptation of Haemophilus influenzae to acquired and innate humoral immunity based on phase variation of lipopolysaccharide. Mol Microbiol 30:767-75.
15. **Swords, W. E., M. L. Moore, L. Godzicki, G. Bukofzer, M. J. Mitten, and J. VonCannon.** 2004. Sialylation of lipooligosaccharides promotes

biofilm formation by nontypeable Haemophilus influenzae. Infect Immun 72:106-13.
16. **Pang, B., D. Winn, R. Johnson, W. Hong, S. West-Barnette, N. Kock, and W. E. Swords.** 2008. Lipooligosaccharides containing phosphorylcholine delay pulmonary clearance of nontypeable Haemophilus influenzae. Infect Immun 76:2037-43.
17. **Wayne, L. G., D. J. Brenner, R. R. Colwell, P. A. D. Grimont, O. Kandler, M. I. Krichevsky, L. H. Moore, W. E. C. Moore, R. G. E. Murray, E. Stackebrandt, M. P. Starr and H. G. Trüper.** 1987. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. Int J Syst Bact 37:463-464.
18. **Konstantinidis, K. T., A. Ramette, and J. M. Tiedje.** 2006. The bacterial species definition in the genomic era. Philos Trans R Soc Lond B Biol Sci 361:1929-40.
19. **Gevers, D., F. M. Cohan, J. G. Lawrence, B. G. Spratt, T. Coenye, E. J. Feil, E. Stackebrandt, Y. Van de Peer, P. Vandamme, F. L. Thompson, and J. Swings.** 2005. Opinion: Re-evaluating prokaryotic species. Nat Rev Microbiol 3:733-9.
20. **Stackebrandt, E., W. Frederiksen, G. M. Garrity, P. A. Grimont, P. Kampfer, M. C. Maiden, X. Nesme, R. Rossello-Mora, J. Swings, H. G. Truper, L. Vauterin, A. C. Ward, and W. B. Whitman.** 2002. Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. Int J Syst Evol Microbiol 52:1043-7.
21. **Kawamura, Y., X. G. Hou, F. Sultana, H. Miura, and T. Ezaki.** 1995. Determination of 16S rRNA sequences of Streptococcus mitis and Streptococcus gordonii and phylogenetic relationships among members of the genus Streptococcus. Int J Syst Bacteriol 45:406-8.
22. **Facklam, R.** 2002. What happened to the streptococci: overview of taxonomic and nomenclature changes. Clin Microbiol Rev 15:613-30.
23. **Kawamura, Y., R. A. Whiley, S. E. Shu, T. Ezaki, and J. M. Hardie.** 1999. Genetic approaches to the identification of the mitis group within the genus Streptococcus. Microbiology 145 (Pt 9):2605-13.
24. **Schloss, P. D., and J. Handelsman.** 2004. Status of the microbial census. Microbiol Mol Biol Rev 68:686-91.
25. **Bluestone, C. D., J. S. Stephenson, and L. M. Martin.** 1992. Ten-year review of otitis media pathogens. Pediatr Infect Dis J 11:S7-11.
26. **Kilpi, T., E. Herva, T. Kaijalainen, R. Syrjanen, and A. K. Takala.** 2001. Bacteriology of acute otitis media in a cohort of Finnish children followed for the first two years of life. Pediatr Infect Dis J 20:654-62.
27. **Sanders, C. V.** 1999. Pneumococcal disease: a symposium in honor of Robert Austrian, MD--a summary. Am J Med 107:86S-90S.
28. **Rudan, I., and H. Campbell.** 2009. The deadly toll of S pneumoniae and H influenzae type b. Lancet 374:854-6.

29. **Faden, H.** 2001. The microbiologic and immunologic basis for recurrent otitis media in children. Eur J Pediatr 160:407-13.
30. **Leibovitz, E., M. R. Jacobs, and R. Dagan.** 2004. Haemophilus influenzae: a significant pathogen in acute otitis media. Pediatr Infect Dis J 23:1142-52.
31. **Finland, M., and M. W. Barnes.** 1977. Changes in occurrence of capsular serotypes of Streptococcus pneumoniae at Boston City Hospital during selected years between 1935 and 1974. J Clin Microbiol 5:154-66.
32. **Martin, M., J. H. Turco, M. E. Zegans, R. R. Facklam, S. Sodha, J. A. Elliott, J. H. Pryor, B. Beall, D. D. Erdman, Y. Y. Baumgartner, P. A. Sanchez, J. D. Schwartzman, J. Montero, A. Schuchat, and C. G. Whitney.** 2003. An outbreak of conjunctivitis due to atypical Streptococcus pneumoniae. N Engl J Med 348:1112-21.
33. **Kadioglu, A., J. N. Weiser, J. C. Paton, and P. W. Andrew.** 2008. The role of Streptococcus pneumoniae virulence factors in host respiratory colonization and disease. Nat Rev Microbiol 6:288-301.
34. **Zissis, N. P., V. Syriopoulou, D. Kafetzis, G. L. Daikos, A. Tsilimingaki, E. Galanakis, and I. Tsangaropoulou.** 2004. Serotype distribution and antimicrobial susceptibility of Streptococcus pneumoniae causing invasive infections and acute otitis media in children. Eur J Pediatr 163:364-8.
35. **Casey, J. R., D. G. Adlowitz, and M. E. Pichichero.** 2009. New Patterns in the Otopathogens Causing Acute Otitis Media Six to Eight Years After Introduction of Pneumococcal Conjugate Vaccine. Pediatr Infect Dis J.
36. **Nelson, A. L., A. M. Roche, J. M. Gould, K. Chim, A. J. Ratner, and J. N. Weiser.** 2007. Capsule enhances pneumococcal colonization by limiting mucus-mediated clearance. Infect Immun 75:83-90.
37. **Yamanaka, N., M. Hotomi, and D. S. Billal.** 2008. Clinical bacteriology and immunology in acute otitis media in children. J Infect Chemother 14:180-7.
38. **Berry, A. M., J. Yother, D. E. Briles, D. Hansman, and J. C. Paton.** 1989. Reduced virulence of a defined pneumolysin-negative mutant of Streptococcus pneumoniae. Infect Immun 57:2037-42.
39. **Walker, J. A., R. L. Allen, P. Falmagne, M. K. Johnson, and G. J. Boulnois.** 1987. Molecular cloning, characterization, and complete nucleotide sequence of the gene for pneumolysin, the sulfhydryl-activated toxin of Streptococcus pneumoniae. Infect Immun 55:1184-9.
40. **Price, K. E., and A. Camilli.** 2009. Pneumolysin localizes to the cell wall of Streptococcus pneumoniae. J Bacteriol 191:2163-8.
41. **Steinfort, C., R. Wilson, T. Mitchell, C. Feldman, A. Rutman, H. Todd, D. Sykes, J. Walker, K. Saunders, P. W. Andrew, and et al.** 1989. Effect of Streptococcus pneumoniae on human respiratory epithelium in vitro. Infect Immun 57:2006-13.
42. **Marriott, H. M., T. J. Mitchell, and D. H. Dockrell.** 2008. Pneumolysin: a double-edged sword during the host-pathogen interaction. Curr Mol Med 8:497-509.

43. **Paton, J. C., B. Rowan-Kelly, and A. Ferrante.** 1984. Activation of human complement by the pneumococcal toxin pneumolysin. Infect Immun 43:1085-7.
44. **Yuste, J., M. Botto, J. C. Paton, D. W. Holden, and J. S. Brown.** 2005. Additive inhibition of complement deposition by pneumolysin and PspA facilitates Streptococcus pneumoniae septicemia. J Immunol 175:1813-9.
45. **Feldman, C., T. J. Mitchell, P. W. Andrew, G. J. Boulnois, R. C. Read, H. C. Todd, P. J. Cole, and R. Wilson.** 1990. The effect of Streptococcus pneumoniae pneumolysin on human respiratory epithelium in vitro. Microb Pathog 9:275-84.
46. **Malley, R., P. Henneke, S. C. Morse, M. J. Cieslewicz, M. Lipsitch, C. M. Thompson, E. Kurt-Jones, J. C. Paton, M. R. Wessels, and D. T. Golenbock.** 2003. Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection. Proc Natl Acad Sci U S A 100:1966-71.
47. **Braun, J. S., J. E. Sublett, D. Freyer, T. J. Mitchell, J. L. Cleveland, E. I. Tuomanen, and J. R. Weber.** 2002. Pneumococcal pneumolysin and H(2)O(2) mediate brain cell apoptosis during meningitis. J Clin Invest 109:19-27.
48. **Martner, A., C. Dahlgren, J. C. Paton, and A. E. Wold.** 2008. Pneumolysin released during Streptococcus pneumoniae autolysis is a potent activator of intracellular oxygen radical production in neutrophils. Infect Immun 76:4079-87.
49. **Holtje, J. V., and A. Tomasz.** 1976. Purification of the pneumococcal N-acetylmuramyl-L-alanine amidase to biochemical homogeneity. J Biol Chem 251:4199-207.
50. **Diaz, E., E. Garcia, C. Ascaso, E. Mendez, R. Lopez, and J. L. Garcia.** 1989. Subcellular localization of the major pneumococcal autolysin: a peculiar mechanism of secretion in Escherichia coli. J Biol Chem 264:1238-44.
51. **Hakenbeck, R., A. Madhour, D. Denapaite, and R. Bruckner.** 2009. Versatility of choline metabolism and choline-binding proteins in Streptococcus pneumoniae and commensal streptococci. FEMS Microbiol Rev 33:572-86.
52. **Sanchez-Puelles, J. M., C. Ronda, J. L. Garcia, P. Garcia, R. Lopez, and E. Garcia.** 1986. Searching for autolysin functions. Characterization of a pneumococcal mutant deleted in the lytA gene. Eur J Biochem 158:289-93.
53. **Berry, A. M., R. A. Lock, D. Hansman, and J. C. Paton.** 1989. Contribution of autolysin to virulence of Streptococcus pneumoniae. Infect Immun 57:2324-30.
54. **Hirst, R. A., B. Gosai, A. Rutman, C. J. Guerin, P. Nicotera, P. W. Andrew, and C. O'Callaghan.** 2008. Streptococcus pneumoniae deficient in pneumolysin or autolysin has reduced virulence in meningitis. J Infect Dis 197:744-51.

55. **Canvin, J. R., A. P. Marvin, M. Sivakumaran, J. C. Paton, G. J. Boulnois, P. W. Andrew, and T. J. Mitchell.** 1995. The role of pneumolysin and autolysin in the pathology of pneumonia and septicemia in mice infected with a type 2 pneumococcus. J Infect Dis 172:119-23.
56. **Tomasz, A., P. Moreillon, and G. Pozzi.** 1988. Insertional inactivation of the major autolysin gene of Streptococcus pneumoniae. J Bacteriol 170:5931-4.
57. **Guiral, S., T. J. Mitchell, B. Martin, and J. P. Claverys.** 2005. Competence-programmed predation of noncompetent cells in the human pathogen Streptococcus pneumoniae: genetic requirements. Proc Natl Acad Sci U S A 102:8710-5.
58. **Balachandran, P., S. K. Hollingshead, J. C. Paton, and D. E. Briles.** 2001. The autolytic enzyme LytA of Streptococcus pneumoniae is not responsible for releasing pneumolysin. J Bacteriol 183:3108-16.
59. **Benton, K. A., J. C. Paton, and D. E. Briles.** 1997. Differences in virulence for mice among Streptococcus pneumoniae strains of capsular types 2, 3, 4, 5, and 6 are not attributable to differences in pneumolysin production. Infect Immun 65:1237-44.
60. **Fleischmann, R. D., M. D. Adams, O. White, R. A. Clayton, E. F. Kirkness, A. R. Kerlavage, C. J. Bult, J. F. Tomb, B. A. Dougherty, J. M. Merrick, and et al.** 1995. Whole-genome random sequencing and assembly of Haemophilus influenzae Rd. Science 269:496-512.
61. **Adams, W. G., K. A. Deaver, S. L. Cochi, B. D. Plikaytis, E. R. Zell, C. V. Broome, and J. D. Wenger.** 1993. Decline of childhood Haemophilus influenzae type b (Hib) disease in the Hib vaccine era. Jama 269:221-6.
62. **Block, S. L., J. Hedrick, C. J. Harrison, R. Tyler, A. Smith, R. Findlay, and E. Keegan.** 2004. Community-wide vaccination with the heptavalent pneumococcal conjugate significantly alters the microbiology of acute otitis media. Pediatr Infect Dis J 23:829-33.
63. **Bouchet, V., D. W. Hood, J. Li, J. R. Brisson, G. A. Randle, A. Martin, Z. Li, R. Goldstein, E. K. Schweda, S. I. Pelton, J. C. Richards, and E. R. Moxon.** 2003. Host-derived sialic acid is incorporated into Haemophilus influenzae lipopolysaccharide and is a major virulence factor in experimental otitis media. Proc Natl Acad Sci U S A 100:8898-903.
64. **Tong, H. H., L. E. Blue, M. A. James, Y. P. Chen, and T. F. DeMaria.** 2000. Evaluation of phase variation of nontypeable Haemophilus influenzae lipooligosaccharide during nasopharyngeal colonization and development of otitis media in the chinchilla model. Infect Immun 68:4593-7.
65. **Janson, H., B. Carl n, A. Cervin, A. Forsgren, A. B. Magnusdottir, S. Lindberg, and T. Runer.** 1999. Effects on the ciliated epithelium of protein D-producing and -nonproducing nontypeable Haemophilus influenzae in nasopharyngeal tissue cultures. J Infect Dis 180:737-46.
66. **Janson, H., A. Melhus, A. Hermansson, and A. Forsgren.** 1994. Protein D, the glycerophosphodiester phosphodiesterase from Haemophilus

influenzae with affinity for human immunoglobulin D, influences virulence in a rat otitis model. Infect Immun 62:4848-54.
67. **Forsgren, A., K. Riesbeck, and H. Janson.** 2008. Protein D of Haemophilus influenzae: a protective nontypeable H. influenzae antigen and a carrier for pneumococcal conjugate vaccines. Clin Infect Dis 46:726-31.
68. **Prymula, R., P. Peeters, V. Chrobok, P. Kriz, E. Novakova, E. Kaliskova, I. Kohl, P. Lommel, J. Poolman, J. P. Prieels, and L. Schuerman.** 2006. Pneumococcal capsular polysaccharides conjugated to protein D for prevention of acute otitis media caused by both Streptococcus pneumoniae and non-typable Haemophilus influenzae: a randomised double-blind efficacy study. Lancet 367:740-8.
69. **Forsgren, J., A. Samuelson, A. Ahlin, J. Jonasson, B. Rynnel-Dagoo, and A. Lindberg.** 1994. Haemophilus influenzae resides and multiplies intracellularly in human adenoid tissue as demonstrated by in situ hybridization and bacterial viability assay. Infect Immun 62:673-9.
70. **Ahren, I. L., H. Janson, A. Forsgren, and K. Riesbeck.** 2001. Protein D expression promotes the adherence and internalization of non-typeable Haemophilus influenzae into human monocytic cells. Microb Pathog 31:151-8.
71. **Verduin, C. M., C. Hol, A. Fleer, H. van Dijk, and A. van Belkum.** 2002. Moraxella catarrhalis: from emerging to established pathogen. Clin Microbiol Rev 15:125-44.
72. **Broides, A., R. Dagan, D. Greenberg, N. Givon-Lavi, and E. Leibovitz.** 2009. Acute otitis media caused by Moraxella catarrhalis: epidemiologic and clinical characteristics. Clin Infect Dis 49:1641-7.
73. **Revai, K., D. P. McCormick, J. Patel, J. J. Grady, K. Saeed, and T. Chonmaitree.** 2006. Effect of pneumococcal conjugate vaccine on nasopharyngeal bacterial colonization during acute otitis media. Pediatrics 117:1823-9.
74. **Vaneechoutte, M., G. Verschraegen, G. Claeys, and A. M. Van Den Abeele.** 1990. Serological typing of Branhamella catarrhalis strains on the basis of lipopolysaccharide antigens. J Clin Microbiol 28:182-7.
75. **Verhaegh, S. J., A. Streefland, J. K. Dewnarain, D. J. Farrell, A. van Belkum, and J. P. Hays.** 2008. Age-related genotypic and phenotypic differences in Moraxella catarrhalis isolates from children and adults presenting with respiratory disease in 2001-2002. Microbiology 154:1178-84.
76. **Bootsma, H. J., H. G. van der Heide, S. van de Pas, L. M. Schouls, and F. R. Mooi.** 2000. Analysis of Moraxella catarrhalis by DNA typing: evidence for a distinct subpopulation associated with virulence traits. J Infect Dis 181:1376-87.
77. **Wirth, T., G. Morelli, B. Kusecek, A. van Belkum, C. van der Schee, A. Meyer, and M. Achtman.** 2007. The rise and spread of a new pathogen: seroresistant Moraxella catarrhalis. Genome Res 17:1647-56.

78. **Nordstrom, T., A. M. Blom, T. T. Tan, A. Forsgren, and K. Riesbeck.** 2005. Ionic binding of C3 to the human pathogen Moraxella catarrhalis is a unique mechanism for combating innate immunity. J Immunol 175:3628-36.
79. **Attia, A. S., S. Ram, P. A. Rice, and E. J. Hansen.** 2006. Binding of vitronectin by the Moraxella catarrhalis UspA2 protein interferes with late stages of the complement cascade. Infect Immun 74:1597-611.
80. **Ali, M. Y.** 1965. Histology of the human nasopharyngeal mucosa. J Anat 99:657-72.
81. **Emerick, K. S., and M. J. Cunningham.** 2006. Tubal tonsil hypertrophy: a cause of recurrent symptoms after adenoidectomy. Arch Otolaryngol Head Neck Surg 132:153-6.
82. **Brook, I., and A. E. Gober.** 2000. In vitro bacterial interference in the nasopharynx of otitis media-prone and non-otitis media-prone children. Arch Otolaryngol Head Neck Surg 126:1011-3.
83. **Brook, I.** 1981. Aerobic and anaerobic bacteriology of adenoids in children: a comparison between patients with chronic adenotonsillitis and adenoid hypertrophy. Laryngoscope 91:377-82.
84. **Raymond, J., I. Le Thomas, F. Moulin, A. Commeau, D. Gendrel, and P. Berche.** 2000. Sequential colonization by Streptococcus pneumoniae of healthy children living in an orphanage. J Infect Dis 181:1983-8.
85. **Raymond, J., L. Armand-Lefevre, F. Moulin, H. Dabernat, A. Commeau, D. Gendrel, and P. Berche.** 2001. Nasopharyngeal colonization by Haemophilus influenzae in children living in an orphanage. Pediatr Infect Dis J 20:779-84.
86. **Hammerschmidt, S., S. Wolff, A. Hocke, S. Rosseau, E. Muller, and M. Rohde.** 2005. Illustration of pneumococcal polysaccharide capsule during adherence and invasion of epithelial cells. Infect Immun 73:4653-67.
87. **Weiser, J. N., R. Austrian, P. K. Sreenivasan, and H. R. Masure.** 1994. Phase variation in pneumococcal opacity: relationship between colonial morphology and nasopharyngeal colonization. Infect Immun 62:2582-9.
88. **Kim, J. O., and J. N. Weiser.** 1998. Association of intrastrain phase variation in quantity of capsular polysaccharide and teichoic acid with the virulence of Streptococcus pneumoniae. J Infect Dis 177:368-77.
89. **Stenfors, L. E., and S. Raisanen.** 1990. Age-dependent changes in bacterial adherence to epithelial cells of nasopharynx in vivo. Acta Otolaryngol 110:292-9.
90. **Heiniger, N., V. Spaniol, R. Troller, M. Vischer, and C. Aebi.** 2007. A reservoir of Moraxella catarrhalis in human pharyngeal lymphoid tissue. J Infect Dis 196:1080-7.
91. **Gould, J. M., and J. N. Weiser.** 2002. The inhibitory effect of C-reactive protein on bacterial phosphorylcholine platelet-activating factor receptor-mediated adherence is blocked by surfactant. J Infect Dis 186:361-71.
92. **Bergmann, S., and S. Hammerschmidt.** 2006. Versatility of pneumococcal surface proteins. Microbiology 152:295-303.

93. **Cundell, D. R., N. P. Gerard, C. Gerard, I. Idanpaan-Heikkila, and E. I. Tuomanen.** 1995. Streptococcus pneumoniae anchor to activated human cells by the receptor for platelet-activating factor. Nature 377:435-8.
94. **Bogaert, D., R. De Groot, and P. W. Hermans.** 2004. Streptococcus pneumoniae colonisation: the key to pneumococcal disease. Lancet Infect Dis 4:144-54.
95. **Murphy, T. F., and G. I. Parameswaran.** 2009. Moraxella catarrhalis, a human respiratory tract pathogen. Clin Infect Dis 49:124-31.
96. **Poolman, J. T., L. Bakaletz, A. Cripps, P. A. Denoel, A. Forsgren, J. Kyd, and Y. Lobet.** 2000. Developing a nontypeable Haemophilus influenzae (NTHi) vaccine. Vaccine 19 Suppl 1:S108-15.
97. **Ronander, E., M. Brant, E. Eriksson, M. Morgelin, O. Hallgren, G. Westergren-Thorsson, A. Forsgren, and K. Riesbeck.** 2009. Nontypeable Haemophilus influenzae adhesin protein E: characterization and biological activity. J Infect Dis 199:522-31.
98. **St Geme, J. W., 3rd, and S. Falkow.** 1990. Haemophilus influenzae adheres to and enters cultured human epithelial cells. Infect Immun 58:4036-44.
99. **Slevogt, H., J. Seybold, K. N. Tiwari, A. C. Hocke, C. Jonatat, S. Dietel, S. Hippenstiel, B. B. Singer, S. Bachmann, N. Suttorp, and B. Opitz.** 2007. Moraxella catarrhalis is internalized in respiratory epithelial cells by a trigger-like mechanism and initiates a TLR2- and partly NOD1-dependent inflammatory immune response. Cell Microbiol 9:694-707.
100. **Jack, R. W., J. R. Tagg, and B. Ray.** 1995. Bacteriocins of gram-positive bacteria. Microbiol Rev 59:171-200.
101. **Tano, K., C. Olofsson, E. Grahn-Hakansson, and S. E. Holm.** 1999. In vitro inhibition of S. pneumoniae, nontypable H. influenzae and M. catharralis by alpha-hemolytic streptococci from healthy children. Int J Pediatr Otorhinolaryngol 47:49-56.
102. **Lim, D. J., J. M. Coticchia, K. Ueno, F. A. Heiselman, and L. O. Bakaletz.** 1991. Glycoconjugates in the chinchilla tubotympanum. Ann Otol Rhinol Laryngol 100:933-43.
103. **Caye-Thomasen, P., and M. Tos.** 2004. Eustachian tube gland tissue changes are related to bacterial species in acute otitis media. Int J Pediatr Otorhinolaryngol 68:101-10.
104. **Corbeel, L.** 2007. What is new in otitis media? Eur J Pediatr 166:511-9.
105. **Bylander, A., A. Ivarsson, and O. Tjernstrom.** 1981. Eustachian tube function in normal children and adults. Acta Otolaryngol 92:481-91.
106. **Bylander, A., and O. Tjernstrom.** 1983. Changes in Eustachian tube function with age in children with normal ears. A longitudinal study. Acta Otolaryngol 96:467-77.
107. **Heikkinen, T., and T. Chonmaitree.** 2003. Importance of respiratory viruses in acute otitis media. Clin Microbiol Rev 16:230-41.

108. **Lim, D. J., Y. M. Chun, H. Y. Lee, S. K. Moon, K. H. Chang, J. D. Li, and A. Andalibi.** 2000. Cell biology of tubotympanum in relation to pathogenesis of otitis media - a review. Vaccine 19 Suppl 1:S17-25.
109. **Vieira, O. V., R. J. Botelho, and S. Grinstein.** 2002. Phagosome maturation: aging gracefully. Biochem J 366:689-704.
110. **Gagnon, E., S. Duclos, C. Rondeau, E. Chevet, P. H. Cameron, O. Steele-Mortimer, J. Paiement, J. J. Bergeron, and M. Desjardins.** 2002. Endoplasmic reticulum-mediated phagocytosis is a mechanism of entry into macrophages. Cell 110:119-31.
111. **Scott, C. C., R. J. Botelho, and S. Grinstein.** 2003. Phagosome maturation: a few bugs in the system. J Membr Biol 193:137-52.
112. **Gruenberg, J.** 2001. The endocytic pathway: a mosaic of domains. Nat Rev Mol Cell Biol 2:721-30.
113. **Ezekowitz, R. A., K. Sastry, P. Bailly, and A. Warner.** 1990. Molecular characterization of the human macrophage mannose receptor: demonstration of multiple carbohydrate recognition-like domains and phagocytosis of yeasts in Cos-1 cells. J Exp Med 172:1785-94.
114. **van der Laan, L. J., E. A. Dopp, R. Haworth, T. Pikkarainen, M. Kangas, O. Elomaa, C. D. Dijkstra, S. Gordon, K. Tryggvason, and G. Kraal.** 1999. Regulation and functional involvement of macrophage scavenger receptor MARCO in clearance of bacteria in vivo. J Immunol 162:939-47.
115. **Underhill, D. M., and A. Ozinsky.** 2002. Phagocytosis of microbes: complexity in action. Annu Rev Immunol 20:825-52.
116. **Martinon, F., A. Mayor, and J. Tschopp.** 2009. The inflammasomes: guardians of the body. Annu Rev Immunol 27:229-65.
117. **Black, R. A., C. T. Rauch, C. J. Kozlosky, J. J. Peschon, J. L. Slack, M. F. Wolfson, B. J. Castner, K. L. Stocking, P. Reddy, S. Srinivasan, N. Nelson, N. Boiani, K. A. Schooley, M. Gerhart, R. Davis, J. N. Fitzner, R. S. Johnson, R. J. Paxton, C. J. March, and D. P. Cerretti.** 1997. A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. Nature 385:729-33.
118. **McHale, J. F., O. A. Harari, D. Marshall, and D. O. Haskard.** 1999. TNF-alpha and IL-1 sequentially induce endothelial ICAM-1 and VCAM-1 expression in MRL/lpr lupus-prone mice. J Immunol 163:3993-4000.
119. **Montgomery, K. F., L. Osborn, C. Hession, R. Tizard, D. Goff, C. Vassallo, P. I. Tarr, K. Bomsztyk, R. Lobb, J. M. Harlan, and et al.** 1991. Activation of endothelial-leukocyte adhesion molecule 1 (ELAM-1) gene transcription. Proc Natl Acad Sci U S A 88:6523-7.
120. **Dinarello, C. A.** 2005. Interleukin-1beta. Crit Care Med 33:S460-2.
121. **Shalaby, M. R., J. Halgunset, O. A. Haugen, H. Aarset, L. Aarden, A. Waage, K. Matsushima, H. Kvithyll, D. Boraschi, J. Lamvik, and et al.** 1991. Cytokine-associated tissue injury and lethality in mice: a comparative study. Clin Immunol Immunopathol 61:69-82.

122. **Yoshimura, T., K. Matsushima, J. J. Oppenheim, and E. J. Leonard.** 1987. Neutrophil chemotactic factor produced by lipopolysaccharide (LPS)-stimulated human blood mononuclear leukocytes: partial characterization and separation from interleukin 1 (IL 1). J Immunol 139:788-93.
123. **Mukaida, N., A. Harada, and K. Matsushima.** 1998. Interleukin-8 (IL-8) and monocyte chemotactic and activating factor (MCAF/MCP-1), chemokines essentially involved in inflammatory and immune reactions. Cytokine Growth Factor Rev 9:9-23.
124. **Baggiolini, M., and I. Clark-Lewis.** 1992. Interleukin-8, a chemotactic and inflammatory cytokine. FEBS Lett 307:97-101.
125. **Davies, P., P. J. Bailey, M. M. Goldenberg, and A. W. Ford-Hutchinson.** 1984. The role of arachidonic acid oxygenation products in pain and inflammation. Annu Rev Immunol 2:335-57.
126. **van der Pouw Kraan, T. C., L. C. Boeije, R. J. Smeenk, J. Wijdenes, and L. A. Aarden.** 1995. Prostaglandin-E2 is a potent inhibitor of human interleukin 12 production. J Exp Med 181:775-9.
127. **Di Battista, J. A., J. Martel-Pelletier, and J. Pelletier.** 1999. Suppression of tumor necrosis factor (TNF-alpha) gene expression by prostaglandin E(2). Role Of early growth response protein-1 (Egr-1). Osteoarthritis Cartilage 7:395-8.
128. **Aloisi, F., R. De Simone, S. Columba-Cabezas, and G. Levi.** 1999. Opposite effects of interferon-gamma and prostaglandin E2 on tumor necrosis factor and interleukin-10 production in microglia: a regulatory loop controlling microglia pro- and anti-inflammatory activities. J Neurosci Res 56:571-80.
129. **Heinrich, P. C., J. V. Castell, and T. Andus.** 1990. Interleukin-6 and the acute phase response. Biochem J 265:621-36.
130. **Jones, S. A.** 2005. Directing transition from innate to acquired immunity: defining a role for IL-6. J Immunol 175:3463-8.
131. **Kaplanski, G., V. Marin, F. Montero-Julian, A. Mantovani, and C. Farnarier.** 2003. IL-6: a regulator of the transition from neutrophil to monocyte recruitment during inflammation. Trends Immunol 24:25-9.
132. **Bevilacqua, M. P., J. S. Pober, M. E. Wheeler, R. S. Cotran, and M. A. Gimbrone, Jr.** 1985. Interleukin 1 acts on cultured human vascular endothelium to increase the adhesion of polymorphonuclear leukocytes, monocytes, and related leukocyte cell lines. J Clin Invest 76:2003-11.
133. **Segal, A. W.** 2005. How neutrophils kill microbes. Annu Rev Immunol 23:197-223.
134. **Korkmaz, B., T. Moreau, and F. Gauthier.** 2008. Neutrophil elastase, proteinase 3 and cathepsin G: physicochemical properties, activity and physiopathological functions. Biochimie 90:227-42.
135. **Woessner, J. F., Jr.** 1991. Matrix metalloproteinases and their inhibitors in connective tissue remodeling. Faseb J 5:2145-54.

136. **Kawai, T., and S. Akira.** 2009. The roles of TLRs, RLRs and NLRs in pathogen recognition. Int Immunol 21:317-37.
137. **Underhill, D. M., A. Ozinsky, A. M. Hajjar, A. Stevens, C. B. Wilson, M. Bassetti, and A. Aderem.** 1999. The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens. Nature 401:811-5.
138. **Ahmad-Nejad, P., H. Hacker, M. Rutz, S. Bauer, R. M. Vabulas, and H. Wagner.** 2002. Bacterial CpG-DNA and lipopolysaccharides activate Toll-like receptors at distinct cellular compartments. Eur J Immunol 32:1958-68.
139. **Ozinsky, A., D. M. Underhill, J. D. Fontenot, A. M. Hajjar, K. D. Smith, C. B. Wilson, L. Schroeder, and A. Aderem.** 2000. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. Proc Natl Acad Sci U S A 97:13766-71.
140. **Wetzler, L. M.** 2003. The role of Toll-like receptor 2 in microbial disease and immunity. Vaccine 21 Suppl 2:S55-60.
141. **Aliprantis, A. O., R. B. Yang, M. R. Mark, S. Suggett, B. Devaux, J. D. Radolf, G. R. Klimpel, P. Godowski, and A. Zychlinsky.** 1999. Cell activation and apoptosis by bacterial lipoproteins through toll-like receptor-2. Science 285:736-9.
142. **Dziarski, R., and D. Gupta.** 2005. Staphylococcus aureus peptidoglycan is a toll-like receptor 2 activator: a reevaluation. Infect Immun 73:5212-6.
143. **Zahringer, U., B. Lindner, S. Inamura, H. Heine, and C. Alexander.** 2008. TLR2 - promiscuous or specific? A critical re-evaluation of a receptor expressing apparent broad specificity. Immunobiology 213:205-24.
144. **Hashimoto, M., K. Tawaratsumida, H. Kariya, A. Kiyohara, Y. Suda, F. Krikae, T. Kirikae, and F. Gotz.** 2006. Not lipoteichoic acid but lipoproteins appear to be the dominant immunobiologically active compounds in Staphylococcus aureus. J Immunol 177:3162-9.
145. **Zhang, G., and S. Ghosh.** 2001. Toll-like receptor-mediated NF-kappaB activation: a phylogenetically conserved paradigm in innate immunity. J Clin Invest 107:13-9.
146. **Elson, G., I. Dunn-Siegrist, B. Daubeuf, and J. Pugin.** 2007. Contribution of Toll-like receptors to the innate immune response to Gram-negative and Gram-positive bacteria. Blood 109:1574-83.
147. **Asplin, I. R., D. J. Carl, S. S. Way, and A. L. Jones.** 2008. Role of Toll-like receptor 2 in innate resistance to Group B Streptococcus. Microb Pathog 44:43-51.
148. **Torres, D., M. Barrier, F. Bihl, V. J. Quesniaux, I. Maillet, S. Akira, B. Ryffel, and F. Erard.** 2004. Toll-like receptor 2 is required for optimal control of Listeria monocytogenes infection. Infect Immun 72:2131-9.
149. **Echchannaoui, H., K. Frei, C. Schnell, S. L. Leib, W. Zimmerli, and R. Landmann.** 2002. Toll-like receptor 2-deficient mice are highly susceptible

to Streptococcus pneumoniae meningitis because of reduced bacterial clearing and enhanced inflammation. J Infect Dis 186:798-806.
150. **Koedel, U., B. Angele, T. Rupprecht, H. Wagner, A. Roggenkamp, H. W. Pfister, and C. J. Kirschning.** 2003. Toll-like receptor 2 participates in mediation of immune response in experimental pneumococcal meningitis. J Immunol 170:438-44.
151. **van Rossum, A. M., E. S. Lysenko, and J. N. Weiser.** 2005. Host and bacterial factors contributing to the clearance of colonization by Streptococcus pneumoniae in a murine model. Infect Immun 73:7718-26.
152. **Knapp, S., C. W. Wieland, C. van 't Veer, O. Takeuchi, S. Akira, S. Florquin, and T. van der Poll.** 2004. Toll-like receptor 2 plays a role in the early inflammatory response to murine pneumococcal pneumonia but does not contribute to antibacterial defense. J Immunol 172:3132-8.
153. **Schumann, R. R., S. R. Leong, G. W. Flaggs, P. W. Gray, S. D. Wright, J. C. Mathison, P. S. Tobias, and R. J. Ulevitch.** 1990. Structure and function of lipopolysaccharide binding protein. Science 249:1429-31.
154. **Triantafilou, M., and K. Triantafilou.** 2002. Lipopolysaccharide recognition: CD14, TLRs and the LPS-activation cluster. Trends Immunol 23:301-4.
155. **Shimazu, R., S. Akashi, H. Ogata, Y. Nagai, K. Fukudome, K. Miyake, and M. Kimoto.** 1999. MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. J Exp Med 189:1777-82.
156. **Uronen-Hansson, H., J. Allen, M. Osman, G. Squires, N. Klein, and R. E. Callard.** 2004. Toll-like receptor 2 (TLR2) and TLR4 are present inside human dendritic cells, associated with microtubules and the Golgi apparatus but are not detectable on the cell surface: integrity of microtubules is required for interleukin-12 production in response to internalized bacteria. Immunology 111:173-8.
157. **Franchi, L., N. Warner, K. Viani, and G. Nunez.** 2009. Function of Nod-like receptors in microbial recognition and host defense. Immunol Rev 227:106-28.
158. **Tattoli, I., L. H. Travassos, L. A. Carneiro, J. G. Magalhaes, and S. E. Girardin.** 2007. The Nodosome: Nod1 and Nod2 control bacterial infections and inflammation. Semin Immunopathol 29:289-301.
159. **McCarthy, J. V., J. Ni, and V. M. Dixit.** 1998. RIP2 is a novel NF-kappaB-activating and cell death-inducing kinase. J Biol Chem 273:16968-75.
160. **Navas, T. A., D. T. Baldwin, and T. A. Stewart.** 1999. RIP2 is a Raf1-activated mitogen-activated protein kinase kinase. J Biol Chem 274:33684-90.
161. **Ogura, Y., N. Inohara, A. Benito, F. F. Chen, S. Yamaoka, and G. Nunez.** 2001. Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-kappaB. J Biol Chem 276:4812-8.
162. **Uehara, A., Y. Sugawara, S. Kurata, Y. Fujimoto, K. Fukase, S. Kusumoto, Y. Satta, T. Sasano, S. Sugawara, and H. Takada.** 2005.

Chemically synthesized pathogen-associated molecular patterns increase the expression of peptidoglycan recognition proteins via toll-like receptors, NOD1 and NOD2 in human oral epithelial cells. Cell Microbiol 7:675-86.

163. **Girardin, S. E., I. G. Boneca, L. A. Carneiro, A. Antignac, M. Jehanno, J. Viala, K. Tedin, M. K. Taha, A. Labigne, U. Zahringer, A. J. Coyle, P. S. DiStefano, J. Bertin, P. J. Sansonetti, and D. J. Philpott.** 2003. Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. Science 300:1584-7.
164. **Chamaillard, M., M. Hashimoto, Y. Horie, J. Masumoto, S. Qiu, L. Saab, Y. Ogura, A. Kawasaki, K. Fukase, S. Kusumoto, M. A. Valvano, S. J. Foster, T. W. Mak, G. Nunez, and N. Inohara.** 2003. An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. Nat Immunol 4:702-7.
165. **Girardin, S. E., I. G. Boneca, J. Viala, M. Chamaillard, A. Labigne, G. Thomas, D. J. Philpott, and P. J. Sansonetti.** 2003. Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. J Biol Chem 278:8869-72.
166. **Inohara, N., Y. Ogura, A. Fontalba, O. Gutierrez, F. Pons, J. Crespo, K. Fukase, S. Inamura, S. Kusumoto, M. Hashimoto, S. J. Foster, A. P. Moran, J. L. Fernandez-Luna, and G. Nunez.** 2003. Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease. J Biol Chem 278:5509-12.
167. **Chamaillard, M., S. E. Girardin, J. Viala, and D. J. Philpott.** 2003. Nods, Nalps and Naip: intracellular regulators of bacterial-induced inflammation. Cell Microbiol 5:581-92.
168. **Kummer, J. A., R. Broekhuizen, H. Everett, L. Agostini, L. Kuijk, F. Martinon, R. van Bruggen, and J. Tschopp.** 2007. Inflammasome components NALP 1 and 3 show distinct but separate expression profiles in human tissues suggesting a site-specific role in the inflammatory response. J Histochem Cytochem 55:443-52.
169. **Martinon, F., K. Burns, and J. Tschopp.** 2002. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. Mol Cell 10:417-26.
170. **Duncan, J. A., D. T. Bergstralh, Y. Wang, S. B. Willingham, Z. Ye, A. G. Zimmermann, and J. P. Ting.** 2007. Cryopyrin/NALP3 binds ATP/dATP, is an ATPase, and requires ATP binding to mediate inflammatory signaling. Proc Natl Acad Sci U S A 104:8041-6.
171. **Kostura, M. J., M. J. Tocci, G. Limjuco, J. Chin, P. Cameron, A. G. Hillman, N. A. Chartrain, and J. A. Schmidt.** 1989. Identification of a monocyte specific pre-interleukin 1 beta convertase activity. Proc Natl Acad Sci U S A 86:5227-31.
172. **Kuida, K., J. A. Lippke, G. Ku, M. W. Harding, D. J. Livingston, M. S. Su, and R. A. Flavell.** 1995. Altered cytokine export and apoptosis in mice deficient in interleukin-1 beta converting enzyme. Science 267:2000-3.

173. **Petrilli, V., S. Papin, C. Dostert, A. Mayor, F. Martinon, and J. Tschopp.** 2007. Activation of the NALP3 inflammasome is triggered by low intracellular potassium concentration. Cell Death Differ 14:1583-9.
174. **Franchi, L., T. D. Kanneganti, G. R. Dubyak, and G. Nunez.** 2007. Differential requirement of P2X7 receptor and intracellular K+ for caspase-1 activation induced by intracellular and extracellular bacteria. J Biol Chem 282:18810-8.
175. **Dostert, C., V. Petrilli, R. Van Bruggen, C. Steele, B. T. Mossman, and J. Tschopp.** 2008. Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. Science 320:674-7.
176. **Zhou, R., A. Tardivel, B. Thorens, I. Choi, and J. Tschopp.** Thioredoxin-interacting protein links oxidative stress to inflammasome activation. Nat Immunol 11:136-40.
177. **Cruz, C. M., A. Rinna, H. J. Forman, A. L. Ventura, P. M. Persechini, and D. M. Ojcius.** 2007. ATP activates a reactive oxygen species-dependent oxidative stress response and secretion of proinflammatory cytokines in macrophages. J Biol Chem 282:2871-9.
178. **Kanneganti, T. D., M. Lamkanfi, Y. G. Kim, G. Chen, J. H. Park, L. Franchi, P. Vandenabeele, and G. Nunez.** 2007. Pannexin-1-mediated recognition of bacterial molecules activates the cryopyrin inflammasome independent of Toll-like receptor signaling. Immunity 26:433-43.
179. **Ferrari, D., C. Pizzirani, E. Adinolfi, R. M. Lemoli, A. Curti, M. Idzko, E. Panther, and F. Di Virgilio.** 2006. The P2X7 receptor: a key player in IL-1 processing and release. J Immunol 176:3877-83.
180. **Rynnel-Dagoo, B., and K. Agren.** 2000. The nasopharynx and the middle ear. Inflammatory reactions in middle ear disease. Vaccine 19 Suppl 1:S26-31.
181. **Brandtzaeg, P.** 2009. Mucosal immunity: induction, dissemination, and effector functions. Scand J Immunol 70:505-15.
182. **Johansen, F. E., E. S. Baekkevold, H. S. Carlsen, I. N. Farstad, D. Soler, and P. Brandtzaeg.** 2005. Regional induction of adhesion molecules and chemokine receptors explains disparate homing of human B cells to systemic and mucosal effector sites: dispersion from tonsils. Blood 106:593-600.
183. **Kobayashi, M., L. Fitz, M. Ryan, R. M. Hewick, S. C. Clark, S. Chan, R. Loudon, F. Sherman, B. Perussia, and G. Trinchieri.** 1989. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. J Exp Med 170:827-45.
184. **D'Andrea, A., M. Rengaraju, N. M. Valiante, J. Chehimi, M. Kubin, M. Aste, S. H. Chan, M. Kobayashi, D. Young, E. Nickbarg, and et al.** 1992. Production of natural killer cell stimulatory factor (interleukin 12) by peripheral blood mononuclear cells. J Exp Med 176:1387-98.

185. **Watford, W. T., M. Moriguchi, A. Morinobu, and J. J. O'Shea.** 2003. The biology of IL-12: coordinating innate and adaptive immune responses. Cytokine Growth Factor Rev 14:361-8.
186. **Trinchieri, G.** 2003. Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat Rev Immunol 3:133-46.
187. **Philip, R., and L. B. Epstein.** 1986. Tumour necrosis factor as immunomodulator and mediator of monocyte cytotoxicity induced by itself, gamma-interferon and interleukin-1. Nature 323:86-9.
188. **Moore, K. W., R. de Waal Malefyt, R. L. Coffman, and A. O'Garra.** 2001. Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol 19:683-765.
189. **Conti, P., D. Kempuraj, K. Kandere, M. D. Gioacchino, R. C. Barbacane, M. L. Castellani, M. Felaco, W. Boucher, R. Letourneau, and T. C. Theoharides.** 2003. IL-10, an inflammatory/inhibitory cytokine, but not always. Immunol Lett 86:123-9.
190. **D'Andrea, A., M. Aste-Amezaga, N. M. Valiante, X. Ma, M. Kubin, and G. Trinchieri.** 1993. Interleukin 10 (IL-10) inhibits human lymphocyte interferon gamma-production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. J Exp Med 178:1041-8.
191. **Kalinski, P., C. M. Hilkens, A. Snijders, F. G. Snijdewint, and M. L. Kapsenberg.** 1997. Dendritic cells, obtained from peripheral blood precursors in the presence of PGE2, promote Th2 responses. Adv Exp Med Biol 417:363-7.
192. **Tripp, C. S., M. K. Gately, J. Hakimi, P. Ling, and E. R. Unanue.** 1994. Neutralization of IL-12 decreases resistance to Listeria in SCID and C.B-17 mice. Reversal by IFN-gamma. J Immunol 152:1883-7.
193. **Dorman, S. E., and S. M. Holland.** 2000. Interferon-gamma and interleukin-12 pathway defects and human disease. Cytokine Growth Factor Rev 11:321-33.
194. **Zhao, Y. X., and A. Tarkowski.** 1995. Impact of interferon-gamma receptor deficiency on experimental Staphylococcus aureus septicemia and arthritis. J Immunol 155:5736-42.
195. **Cusumano, V., G. Mancuso, F. Genovese, D. Delfino, C. Beninati, E. Losi, and G. Teti.** 1996. Role of gamma interferon in a neonatal mouse model of group B streptococcal disease. Infect Immun 64:2941-4.
196. **Rubins, J. B., and C. Pomeroy.** 1997. Role of gamma interferon in the pathogenesis of bacteremic pneumococcal pneumonia. Infect Immun 65:2975-7.
197. **van der Poll, T., A. Marchant, C. V. Keogh, M. Goldman, and S. F. Lowry.** 1996. Interleukin-10 impairs host defense in murine pneumococcal pneumonia. J Infect Dis 174:994-1000.
198. **Hessle, C., L. A. Hanson, and A. E. Wold.** 1999. Lactobacilli from human gastrointestinal mucosa are strong stimulators of IL-12 production. Clin Exp Immunol 116:276-82.

199. **Barkman, C., A. Martner, C. Hessle, and A. E. Wold.** 2008. Soluble bacterial constituents down-regulate secretion of IL-12 in response to intact Gram-positive bacteria. Microbes Infect 10:1484-93.
200. **Hessle, C., B. Andersson, and A. E. Wold.** 2000. Gram-positive bacteria are potent inducers of monocytic interleukin-12 (IL-12) while gram-negative bacteria preferentially stimulate IL-10 production. Infect Immun 68:3581-6.
201. **Hessle, C. C., B. Andersson, and A. E. Wold.** 2003. Gram-negative, but not Gram-positive, bacteria elicit strong PGE2 production in human monocytes. Inflammation 27:329-32.
202. **Hessle, C. C., B. Andersson, and A. E. Wold.** 2005. Gram-positive and Gram-negative bacteria elicit different patterns of pro-inflammatory cytokines in human monocytes. Cytokine 30:311-8.
203. **Rovers, M. M., A. G. Schilder, G. A. Zielhuis, and R. M. Rosenfeld.** 2004. Otitis media. Lancet 363:465-73.
204. **Palmu, A. A., E. Herva, H. Savolainen, P. Karma, P. H. Makela, and T. M. Kilpi.** 2004. Association of clinical signs and symptoms with bacterial findings in acute otitis media. Clin Infect Dis 38:234-42.
205. **Heikkinen, T., and O. Ruuskanen.** 1994. Temporal development of acute otitis media during upper respiratory tract infection. Pediatr Infect Dis J 13:659-61.
206. **Koivunen, P., T. Kontiokari, M. Niemela, T. Pokka, and M. Uhari.** 1999. Time to development of acute otitis media during an upper respiratory tract infection in children. Pediatr Infect Dis J 18:303-5.
207. **Ruuskanen, O., M. Arola, A. Putto-Laurila, J. Mertsola, O. Meurman, M. K. Viljanen, and P. Halonen.** 1989. Acute otitis media and respiratory virus infections. Pediatr Infect Dis J 8:94-9.
208. **Uhari, M., J. Hietala, and H. Tuokko.** 1995. Risk of acute otitis media in relation to the viral etiology of infections in children. Clin Infect Dis 20:521-4.
209. **Chonmaitree, T., K. Revai, J. J. Grady, A. Clos, J. A. Patel, S. Nair, J. Fan, and K. J. Henrickson.** 2008. Viral upper respiratory tract infection and otitis media complication in young children. Clin Infect Dis 46:815-23.
210. **Peltola, V. T., and J. A. McCullers.** 2004. Respiratory viruses predisposing to bacterial infections: role of neuraminidase. Pediatr Infect Dis J 23:S87-97.
211. **Nokso-Koivisto, J., T. Hovi, and A. Pitkaranta.** 2006. Viral upper respiratory tract infections in young children with emphasis on acute otitis media. Int J Pediatr Otorhinolaryngol 70:1333-42.
212. **Abramson, J. S., G. S. Giebink, and P. G. Quie.** 1982. Influenza A virus-induced polymorphonuclear leukocyte dysfunction in the pathogenesis of experimental pneumococcal otitis media. Infect Immun 36:289-96.
213. **Giebink, G. S., M. L. Ripley, and P. F. Wright.** 1987. Eustachian tube histopathology during experimental influenza A virus infection in the chinchilla. Ann Otol Rhinol Laryngol 96:199-206.

214. **Giebink, G. S., I. K. Berzins, S. C. Marker, and G. Schiffman.** 1980. Experimental otitis media after nasal inoculation of Streptococcus pneumoniae and influenza A virus in chinchillas. Infect Immun 30:445-50.
215. **Brook, I., and P. Yocum.** 1999. Bacterial interference in the adenoids of otitis media-prone children. Pediatr Infect Dis J 18:835-7.
216. **Faden, H., L. Duffy, R. Wasielewski, J. Wolf, D. Krystofik, and Y. Tung.** 1997. Relationship between nasopharyngeal colonization and the development of otitis media in children. Tonawanda/Williamsville Pediatrics. J Infect Dis 175:1440-5.
217. **Bernstein, J. M., S. Sagahtaheri-Altaie, D. M. Dryja, and J. Wactawski-Wende.** 1994. Bacterial interference in nasopharyngeal bacterial flora of otitis-prone and non-otitis-prone children. Acta Otorhinolaryngol Belg 48:1-9.
218. **Marchisio, P., L. Claut, A. Rognoni, S. Esposito, D. Passali, L. Bellussi, L. Drago, G. Pozzi, S. Mannelli, G. Schito, and N. Principi.** 2003. Differences in nasopharyngeal bacterial flora in children with nonsevere recurrent acute otitis media and chronic otitis media with effusion: implications for management. Pediatr Infect Dis J 22:262-8.
219. **Lindberg, K., B. Rynnel-Dagoo, and K. G. Sundqvist.** 1994. Cytokines in nasopharyngeal secretions; evidence for defective IL-1 beta production in children with recurrent episodes of acute otitis media. Clin Exp Immunol 97:396-402.
220. **Broides, A., E. Leibovitz, R. Dagan, J. Press, S. Raiz, M. Kafka, A. Leiberman, and T. Yermiahu.** 2002. Cytology of middle ear fluid during acute otitis media. Pediatr Infect Dis J 21:57-61.
221. **Goldie, P., S. Hellstrom, and U. Johansson.** 1990. Vascular events in experimental otitis media models: a comparative study. ORL J Otorhinolaryngol Relat Spec 52:104-12.
222. **Caye-Thomasen, P., A. Hermansson, M. Tos, and K. Prellner.** 1995. Changes in goblet cell density in rat middle ear mucosa in acute otitis media. Am J Otol 16:75-82.
223. **Tos, M., and P. Caye-Thomasen.** 2002. Mucous glands in the middle ear - what is known and what is not. ORL J Otorhinolaryngol Relat Spec 64:86-94.
224. **Rodriguez, W. J., and R. H. Schwartz.** 1999. Streptococcus pneumoniae causes otitis media with higher fever and more redness of tympanic membranes than Haemophilus influenzae or Moraxella catarrhalis. Pediatr Infect Dis J 18:942-4.
225. **Karma, P., J. Luotonen, J. Pukander, M. Sipila, E. Herva, and P. Gronroos.** 1983. Haemophilus influenzae in acute otitis media. Acta Otolaryngol 95:105-10.
226. **Brook, I., and A. E. Gober.** 2000. Reliability of the microbiology of spontaneously draining acute otitis media in children. Pediatr Infect Dis J 19:571-3.

227. **Molstad, S., M. Erntell, H. Hanberger, E. Melander, C. Norman, G. Skoog, C. S. Lundborg, A. Soderstrom, E. Torell, and O. Cars.** 2008. Sustained reduction of antibiotic use and low bacterial resistance: 10-year follow-up of the Swedish Strama programme. Lancet Infect Dis 8:125-32.
228. **Barzilai, A., E. Leibovitz, J. H. Laver, L. Piglansky, S. Raiz, M. R. Abboud, D. M. Fliss, A. Leiberman, and R. Dagan.** 1999. Dynamics of interleukin-1 production in middle ear fluid during acute otitis media treated with antibiotics. Infection 27:173-6.
229. **Barzilai, A., B. Dekel, R. Dagan, J. H. Passwell, and E. Leibovitz.** 1999. Cytokine analysis of middle ear effusions during acute otitis media: significant reduction in tumor necrosis factor alpha concentrations correlates with bacterial eradication. Pediatr Infect Dis J 18:301-3.
230. **Leibovitz, E., R. Dagan, J. H. Laver, L. Piglansky, S. Raiz, M. R. Abboud, D. M. Fliss, A. Leiberman, and A. Barzilai.** 2000. Interleukin 8 in middle ear fluid during acute otitis media: correlation with aetiology and bacterial eradication. Arch Dis Child 82:165-8.
231. **Barzilaia, A., B. Dekel, R. Dagan, and E. Leibovitz.** 2000. Middle ear effusion IL-6 concentration in bacterial and non-bacterial acute otitis media. Acta Paediatr 89:1068-71.
232. **Chonmaitree, T., and K. J. Henrickson.** 2000. Detection of respiratory viruses in the middle ear fluids of children with acute otitis media by multiplex reverse transcription:polymerase chain reaction assay. Pediatr Infect Dis J 19:258-60.
233. **Noah, T. L., F. W. Henderson, I. A. Wortman, R. B. Devlin, J. Handy, H. S. Koren, and S. Becker.** 1995. Nasal cytokine production in viral acute upper respiratory infection of childhood. J Infect Dis 171:584-92.
234. **Fritz, R. S., F. G. Hayden, D. P. Calfee, L. M. Cass, A. W. Peng, W. G. Alvord, W. Strober, and S. E. Straus.** 1999. Nasal cytokine and chemokine responses in experimental influenza A virus infection: results of a placebo-controlled trial of intravenous zanamivir treatment. J Infect Dis 180:586-93.
235. **Turner, R. B., K. W. Weingand, C. H. Yeh, and D. W. Leedy.** 1998. Association between interleukin-8 concentration in nasal secretions and severity of symptoms of experimental rhinovirus colds. Clin Infect Dis 26:840-6.
236. **Patel, J. A., S. Nair, K. Revai, J. Grady, and T. Chonmaitree.** 2009. Nasopharyngeal acute phase cytokines in viral upper respiratory infection: impact on acute otitis media in children. Pediatr Infect Dis J 28:1002-7.
237. **Kleemola, M., J. Nokso-Koivisto, E. Herva, R. Syrjanen, M. Lahdenkari, T. Kilpi, and T. Hovi.** 2006. Is there any specific association between respiratory viruses and bacteria in acute otitis media of young children? J Infect 52:181-7.
238. **Chonmaitree, T., M. J. Owen, and V. M. Howie.** 1990. Respiratory viruses interfere with bacteriologic response to antibiotic in children with acute otitis media. J Infect Dis 162:546-9.

239. **Chonmaitree, T., M. J. Owen, J. A. Patel, D. Hedgpeth, D. Horlick, and V. M. Howie.** 1992. Effect of viral respiratory tract infection on outcome of acute otitis media. J Pediatr 120:856-62.
240. **Jossart, G. H., D. M. Canafax, G. R. Erdmann, M. J. Lovdahl, H. Q. Russlie, S. K. Juhn, and G. S. Giebink.** 1994. Effect of Streptococcus pneumoniae and influenza A virus on middle ear antimicrobial pharmacokinetics in experimental otitis media. Pharm Res 11:860-4.
241. **Canafax, D. M., Z. Yuan, T. Chonmaitree, K. Deka, H. Q. Russlie, and G. S. Giebink.** 1998. Amoxicillin middle ear fluid penetration and pharmacokinetics in children with acute otitis media. Pediatr Infect Dis J 17:149-56.
242. **Chonmaitree, T., J. A. Patel, T. Sim, R. Garofalo, T. Uchida, V. M. Howie, and M. J. Owen.** 1996. Role of leukotriene B4 and interleukin-8 in acute bacterial and viral otitis media. Ann Otol Rhinol Laryngol 105:968-74.
243. **Chonmaitree, T., J. A. Patel, M. A. Lett-Brown, T. Uchida, R. Garofalo, M. J. Owen, and V. M. Howie.** 1994. Virus and bacteria enhance histamine production in middle ear fluids of children with acute otitis media. J Infect Dis 169:1265-70.
244. **Gates, G. A., J. O. Klein, D. J. Lim, G. Mogi, P. L. Ogra, M. M. Pararella, J. L. Paradise, and M. Tos.** 2002. Recent advances in otitis media. 1. Definitions, terminology, and classification of otitis media. Ann Otol Rhinol Laryngol Suppl 188:8-18.
245. **Rosenfeld, R. M., L. Culpepper, K. J. Doyle, K. M. Grundfast, A. Hoberman, M. A. Kenna, A. S. Lieberthal, M. Mahoney, R. A. Wahl, C. R. Woods, Jr., and B. Yawn.** 2004. Clinical practice guideline: Otitis media with effusion. Otolaryngol Head Neck Surg 130:S95-118.
246. **Tos, M., and K. Bak-Pedersen.** 1973. Density of mucous glands in various sequelae to otitis media. J Laryngol Otol 87:1183-92.
247. **Post, J. C., R. A. Preston, J. J. Aul, M. Larkins-Pettigrew, J. Rydquist-White, K. W. Anderson, R. M. Wadowsky, D. R. Reagan, E. S. Walker, L. A. Kingsley, and et al.** 1995. Molecular analysis of bacterial pathogens in otitis media with effusion. Jama 273:1598-604.
248. **Hendolin, P. H., A. Markkanen, J. Ylikoski, and J. J. Wahlfors.** 1997. Use of multiplex PCR for simultaneous detection of four bacterial species in middle ear effusions. J Clin Microbiol 35:2854-8.
249. **Bakaletz, L. O.** 2007. Bacterial biofilms in otitis media: evidence and relevance. Pediatr Infect Dis J 26:S17-9.
250. **Costerton, J. W., P. S. Stewart, and E. P. Greenberg.** 1999. Bacterial biofilms: a common cause of persistent infections. Science 284:1318-22.
251. **Post, J. C., N. L. Hiller, L. Nistico, P. Stoodley, and G. D. Ehrlich.** 2007. The role of biofilms in otolaryngologic infections: update 2007. Curr Opin Otolaryngol Head Neck Surg 15:347-51.
252. **Hall-Stoodley, L., F. Z. Hu, A. Gieseke, L. Nistico, D. Nguyen, J. Hayes, M. Forbes, D. P. Greenberg, B. Dice, A. Burrows, P. A. Wackym, P.**

Stoodley, J. C. Post, G. D. Ehrlich, and J. E. Kerschner. 2006. Direct detection of bacterial biofilms on the middle-ear mucosa of children with chronic otitis media. Jama 296:202-11.

253. **Hong, W., K. Mason, J. Jurcisek, L. Novotny, L. O. Bakaletz, and W. E. Swords.** 2007. Phosphorylcholine decreases early inflammation and promotes the establishment of stable biofilm communities of nontypeable Haemophilus influenzae strain 86-028NP in a chinchilla model of otitis media. Infect Immun 75:958-65.
254. **FAO/WHO.** 2001. Probiotics in food.
255. **Sanders, M. E.** 2003. Probiotics: considerations for human health. Nutr Rev 61:91-9.
256. **Vouloumanou, E. K., G. C. Makris, D. E. Karageorgopoulos, and M. E. Falagas.** 2009. Probiotics for the prevention of respiratory tract infections: a systematic review. Int J Antimicrob Agents 34:197 e1-10.
257. **Leyer, G. J., S. Li, M. E. Mubasher, C. Reifer, and A. C. Ouwehand.** 2009. Probiotic effects on cold and influenza-like symptom incidence and duration in children. Pediatrics 124:e172-9.
258. **Roos, K., S. E. Holm, E. Grahn-Hakansson, and L. Lagergren.** 1996. Recolonization with selected alpha-streptococci for prophylaxis of recurrent streptococcal pharyngotonsillitis--a randomized placebo-controlled multicentre study. Scand J Infect Dis 28:459-62.
259. **Falck, G., E. Grahn-Hakansson, S. E. Holm, K. Roos, and L. Lagergren.** 1999. Tolerance and efficacy of interfering alpha-streptococci in recurrence of streptococcal pharyngotonsillitis: a placebo-controlled study. Acta Otolaryngol 119:944-8.
260. **Roos, K., E. G. Hakansson, and S. Holm.** 2001. Effect of recolonisation with "interfering" alpha streptococci on recurrences of acute and secretory otitis media in children: randomised placebo controlled trial. Bmj 322:210-2.
261. **Karlsson, H., C. Hessle, and A. Rudin.** 2002. Innate immune responses of human neonatal cells to bacteria from the normal gastrointestinal flora. Infect Immun 70:6688-96.
262. **Fritzell, P., T. Bergstrom, and C. Welinder-Olsson.** 2004. Detection of bacterial DNA in painful degenerated spinal discs in patients without signs of clinical infection. Eur Spine J 13:702-6.
263. **Skovbjerg, S., C. Welinder-Olsson, N. Kondori, E. Kjellin, F. Nowrouzian, A. E. Wold, D. Stockelberg, P. Larsson, and C. Wenneras.** 2009. Optimization of the detection of microbes in blood from immunocompromised patients with haematological malignancies. Clin Microbiol Infect 15:680-3.
264. **Brittain-Long, R., S. Nord, S. Olofsson, J. Westin, L. M. Anderson, and M. Lindh.** 2008. Multiplex real-time PCR for detection of respiratory tract infections. J Clin Virol 41:53-6.

265. **Giudicelli, S., and A. Tomasz.** 1984. Attachment of pneumococcal autolysin to wall teichoic acids, an essential step in enzymatic wall degradation. J Bacteriol 158:1188-90.
266. **Boulnois, G. J., J. C. Paton, T. J. Mitchell, and P. W. Andrew.** 1991. Structure and function of pneumolysin, the multifunctional, thiol-activated toxin of Streptococcus pneumoniae. Mol Microbiol 5:2611-6.
267. **Cooper, A. M., J. Magram, J. Ferrante, and I. M. Orme.** 1997. Interleukin 12 (IL-12) is crucial to the development of protective immunity in mice intravenously infected with mycobacterium tuberculosis. J Exp Med 186:39-45.
268. **Heinzel, F. P., D. S. Schoenhaut, R. M. Rerko, L. E. Rosser, and M. K. Gately.** 1993. Recombinant interleukin 12 cures mice infected with Leishmania major. J Exp Med 177:1505-9.
269. **Yamamoto, N., K. Kawakami, Y. Kinjo, K. Miyagi, T. Kinjo, K. Uezu, C. Nakasone, M. Nakamatsu, and A. Saito.** 2004. Essential role for the p40 subunit of interleukin-12 in neutrophil-mediated early host defense against pulmonary infection with Streptococcus pneumoniae: involvement of interferon-gamma. Microbes Infect 6:1241-9.
270. **Weigent, D. A., T. L. Huff, J. W. Peterson, G. J. Stanton, and S. Baron.** 1986. Role of interferon in streptococcal infection in the mouse. Microb Pathog 1:399-407.
271. **O'Brien, D. P., D. E. Briles, A. J. Szalai, A. H. Tu, I. Sanz, and M. H. Nahm.** 1999. Tumor necrosis factor alpha receptor I is important for survival from Streptococcus pneumoniae infections. Infect Immun 67:595-601.
272. **Shida, K., J. Kiyoshima-Shibata, M. Nagaoka, K. Watanabe, and M. Nanno.** 2006. Induction of interleukin-12 by lactobacillus strains having a rigid cell wall resistant to intracellular digestion. J Dairy Sci 89:3306-17.
273. **Masumoto, J., K. Yang, S. Varambally, M. Hasegawa, S. A. Tomlins, S. Qiu, Y. Fujimoto, A. Kawasaki, S. J. Foster, Y. Horie, T. W. Mak, G. Nunez, A. M. Chinnaiyan, K. Fukase, and N. Inohara.** 2006. Nod1 acts as an intracellular receptor to stimulate chemokine production and neutrophil recruitment in vivo. J Exp Med 203:203-13.
274. **Mosa, A., C. Trumstedt, E. Eriksson, O. Soehnlein, F. Heuts, K. Janik, A. Klos, O. Dittrich-Breiholz, M. Kracht, A. Hidmark, H. Wigzell, and M. E. Rottenberg.** 2009. Nonhematopoietic cells control the outcome of infection with Listeria monocytogenes in a nucleotide oligomerization domain 1-dependent manner. Infect Immun 77:2908-18.
275. **Cossart, P., and M. Lecuit.** 1998. Interactions of Listeria monocytogenes with mammalian cells during entry and actin-based movement: bacterial factors, cellular ligands and signaling. Embo J 17:3797-806.
276. **Schnupf, P., and D. A. Portnoy.** 2007. Listeriolysin O: a phagosome-specific lysin. Microbes Infect 9:1176-87.

277. **Warren, S. E., D. P. Mao, A. E. Rodriguez, E. A. Miao, and A. Aderem.** 2008. Multiple Nod-like receptors activate caspase 1 during Listeria monocytogenes infection. J Immunol 180:7558-64.
278. **Janot, L., T. Secher, D. Torres, I. Maillet, J. Pfeilschifter, V. F. Quesniaux, R. Landmann, B. Ryffel, and F. Erard.** 2008. CD14 works with toll-like receptor 2 to contribute to recognition and control of Listeria monocytogenes infection. J Infect Dis 198:115-24.
279. **Smith, T. J., S. A. Blackman, and S. J. Foster.** 2000. Autolysins of Bacillus subtilis: multiple enzymes with multiple functions. Microbiology 146 (Pt 2):249-62.
280. **Raddadi, N., A. Cherif, D. Mora, L. Brusetti, S. Borin, A. Boudabous, and D. Daffonchio.** 2005. The autolytic phenotype of the Bacillus cereus group. J Appl Microbiol 99:1070-81.
281. **Bublitz, M., L. Polle, C. Holland, D. W. Heinz, M. Nimtz, and W. D. Schubert.** 2009. Structural basis for autoinhibition and activation of Auto, a virulence-associated peptidoglycan hydrolase of Listeria monocytogenes. Mol Microbiol 71:1509-22.
282. **Shida, K., J. Kiyoshima-Shibata, R. Kaji, M. Nagaoka, and M. Nanno.** 2009. Peptidoglycan from lactobacilli inhibits interleukin-12 production by macrophages induced by Lactobacillus casei through Toll-like receptor 2-dependent and independent mechanisms. Immunology 128:e858-69.
283. **Watanabe, T., A. Kitani, P. J. Murray, and W. Strober.** 2004. NOD2 is a negative regulator of Toll-like receptor 2-mediated T helper type 1 responses. Nat Immunol 5:800-8.
284. **Siqueira, J. F., Jr., and I. N. Rocas.** 2004. Nested PCR detection of Centipeda periodontii in primary endodontic infections. J Endod 30:135-7.
285. **Kokeguchi, S., O. Tsutsui, K. Kato, and T. Matsumura.** 1990. Isolation and characterization of lipopolysaccharide from Centipeda periodontii ATCC 35019. Oral Microbiol Immunol 5:108-12.
286. **Ulstrup, A. K., and S. H. Hartzen.** 2006. Leptotrichia buccalis: a rare cause of bacteraemia in non-neutropenic patients. Scand J Infect Dis 38:712-6.
287. **Zambon, J. J., C. DeLuca, J. Slots, and R. J. Genco.** 1983. Studies of leukotoxin from Actinobacillus actinomycetemcomitans using the promyelocytic HL-60 cell line. Infect Immun 40:205-12.
288. **Havemose-Poulsen, A., and P. Holmstrup.** 1997. Factors affecting IL-1-mediated collagen metabolism by fibroblasts and the pathogenesis of periodontal disease: a review of the literature. Crit Rev Oral Biol Med 8:217-36.
289. **Schousboe, L. P., T. Ovesen, L. Eckhardt, L. M. Rasmussen, and C. B. Pedersen.** 2001. How does endotoxin trigger inflammation in otitis media with effusion? Laryngoscope 111:297-300.
290. **Forseni, M., A. Melhus, A. F. Ryan, D. Bagger-Sjoback, and M. Hultcrantz.** 2001. Detection and localization of interleukin-6 in the rat middle ear during experimental acute otitis media, using mRNA in situ

hybridization and immunohistochemistry. Int J Pediatr Otorhinolaryngol 57:115-21.
291. **Heikkinen, T., F. Ghaffar, A. O. Okorodudu, and T. Chonmaitree.** 1998. Serum interleukin-6 in bacterial and nonbacterial acute otitis media. Pediatrics 102:296-9.
292. **Cohen, J., and E. Abraham.** 1999. Microbiologic findings and correlations with serum tumor necrosis factor-alpha in patients with severe sepsis and septic shock. J Infect Dis 180:116-21.
293. **Diab, A., J. Zhu, L. Lindquist, B. Wretlind, M. Bakhiet, and H. Link.** 1997. Haemophilus influenzae and Streptococcus pneumoniae induce different intracerebral mRNA cytokine patterns during the course of experimental bacterial meningitis. Clin Exp Immunol 109:233-41.
294. **Brook, I., and A. E. Gober.** 2009. Bacteriology of spontaneously draining acute otitis media in children before and after the introduction of pneumococcal vaccination. Pediatr Infect Dis J 28:640-2.
295. **Leskinen, K., P. Hendolin, A. Virolainen-Julkunen, J. Ylikoski, and J. Jero.** 2004. Alloiococcus otitidis in acute otitis media. Int J Pediatr Otorhinolaryngol 68:51-6.